Universidade de Lisboa

Faculdade de Ciências

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The dual role of enterococci in food technology: bacteriocin production versus pathogenicity potential

Sara Filipa Correia Santos

Mestrado em Microbiologia Aplicada

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2011

“La science n'a pas de patrie, parce que le savoir est le patrimoine de l'humanité, le flambeau qui éclaire le monde.”

Louis Pasteur

Discours d’inauguration de l’Institut Pasteur

14 Novembre 1888

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Abstract

Enterococci are usually isolated from fermented foods, in which they contribute to the ripening/organoleptic characteristics, but nowadays are considered emerging pathogens, due to an increase of antibiotic resistances and production of virulence traits. Although enterococcal foodborne infections have not been described so far, this combination of factors raises special awareness regarding the food safety of products harboring enterococci.

The present study analyzed Portuguese traditional meat fermented products namely, Catalão, Chouriço Preto, Linguiça, Paio and Salsichão, for the presence of *Enterococcus* spp., focusing on their technological interest and pathogenicity potential.

148 enterococci were presumptively identified at genus level by growth in selective media, at different temperatures, pH values, NaCl concentrations and assessment of Gram-staining, catalase and oxidase activities. Subsequently, the genomic diversity of the isolates was assessed by PCR-fingerprinting with primers OPC-19 and $(GTG)_5$ and calculation of Simpson's diversity index (D=0.927). Analysis of the dendrogram obtained led to the selection of 75 meat-enterococci, representative of all enterococcal groups, which were identified by multiplex-PCR as members of the species *Enterococcus faecalis* (n=45), *E. faecium* (n=22) and *E. durans* (n=8).

To evaluate technological potential the meat-enterococci were tested for the production of bacteriocins and enzymes of technological relevance: 8% produced bacteriocins and several enzymatic activities were observed (*e.g* acid phosphatase, cystine arylamidase, lipase and casein).

Subsequently, the meat-enterococci were evaluated for the presence of virulence factors: Genes *agg*, *esp* and *cylA* were absent and the most frequent virulence determinants were *ebpC* 28%, *epa* 28% and *gelE* 75%. Ten of the isolates produced gelatinase and none is β-hemolytic. The isolates were also tested for biofilm production with 39% being considered strong biofilm producers.

The strains were also evaluated for their susceptibility to fourteen antibiotics. All the meat-enterococci were found to be resistant to cefalexin, nalidixic acid, streptomycin and sulphamethoxazol/trimethropim and susceptible to ampicillin, amoxycillin/clavulanate and high level gentamicin. For the remaining agents the following resistances were observed: 3% for chloramphenicol,12% for erythromycin, 71% for gentamicin-10, 36% for penicillin G, 60% for tetracycline and 20% for vancomycin (of which 5 isolates were found to be resistant to high levels MIC>256μg/ml).

Overall, even thought the meat enterococci present several antibiotic resistances and produce biofilms, due to a low number of virulence factors and to the absence of reports regarding foodborne infections, a low risk is probably associated with the presence of enterococci in these long-established traditional meat fermented products.

Key-Words: Fermented meat products, *Enterococcus*, technological interest, pathogenic potential.

Resumo

Os enterococcos são encontrados numa grande variedade de ambientes, tal como em águas ou no solo. Estas bactérias ubíquas fazem parte da microbiota autóctone do tracto gastrointestinal do Homem e diferentes animais de sangue quente (Murray, 1990; Klein, 2003). São ainda frequentemente encontrados em alimentos fermentados, como produtos lácteos e carnes (Franz *et al.*,1999; Hugas *et al.*, 2003). Actualmente, os enterococos são considerados como patogénicos emergentes, devido à sua elevada resistência a antibióticos e produção de factores de virulência (Semedo-Lemsaddek e Mato, 2011). Embora até este momento não tenham sido descritas toxinfecções causadas por enterococos, esta combinação de factores levanta sérias questões de segurança alimentar, especialmente para os produtos a que os enterococcos estão associados.

Em Portugal, bem como noutros países mediterrâneos, o fabrico e consumo de enchidos tradicionais é muito frequente (Barbosa *et al.*, 2009; Ribeiro *et al.*, 2011). Assim, uma vez que os enterococos são frequentemente encontrados em produtos cárneos fermentados, torna-se importante perceber qual a sua frequência nestes produtos e que tipo de potencial patogénico os isolados alimentares apresentam.

A patogenicidade dos enterococos é atribuída à presença/expressão de diversos factores de virulência (e.g. *agg*, *efaAfs*, *esp*, *gelE*, *cylA*, *epa* e *fsrABC*) bem como à resistência (intrínseca ou adquirida) a vários antibióticos. Ainda relacionado com o potencial patogénico, encontra-se a capacidade de produção de biofilmes. Estas formas de resistência ambiental podem permitir a permanência de microrganismos no ambiente fabril, facilitanto o seu estabelecimento nos produtos e a sua disseminação para os consumidores através da cadeia alimentar (Paganelli and Leavis, 2011).

Por outro lado, muitas das características organolépticas dos produtos fermentados são atribuídas à fermentação levada a cabo pelas bactérias lácticas, entre elas aos enterococos (Giraffa, 2002). Deste modo, torna-se também importante perceber qual o interesse para a tecnologia alimentar que os enterococos presentes nos enchidos podem apresentar (que tipo de enzimas produzem; se produzem bacteriocinas; quais são e qual o seu espectro de acção).

Neste estudo, a presença de *Enterococcus* spp. foi analisada em vários produtos cárneos fermentados Portugueses fabricados de forma tradicional. Nomeadamente, Catalão, Chouriço Preto, Linguiça, Paio e Salsichão. No total, foram isolados 148 enterococos de todos os produtos analisados, tendo os mesmos sido presuntivamente identificados ao nível de género através de uma bateria de testes fenotípicos que incluiram o crescimento em dois meios selectivos (Slanetz and Bartley agar e Bílis Esculina Azida agar), a diferentes temperaturas (10, 37 e 45ºC), a pH elevado (9,6) e em presença de concentrações salinas de 6,5%, coloração de Gram, teste de actividade da catalase e oxidase. Paralelamente, para uma identificação mais fidedigna a nível de género, foi ainda efectuado um PCR dirigido para sequências específicas do género *Enterococcus* (Ke *et al.*, 1999).

De seguida, a diversidade da colecção de isolados de enchidos foi avaliada por PCR-fingerprinting utilizando dois primers aleatórios OPC19 e $(GTG)_5$. Com os perfis de bandas obtidos foi construído um dendrograma, a partir do qual foi possível efectuar a selecção de 75 enterococos, representando todos os grupos, para estudos posteriores. Foi ainda calculado o índice de diversidade de Simpson (D=0.927).

Procedeu-se então á identificação dos isolados ao nível de espécie; tendo os mesmos sido incluídos nas espécies *Enterococcus faecalis* (n=45), *E. faecium* (n=22) e *E. durans* (n=8).

Subsequentemente foi avaliada a susceptibilidade dos isolados a catorze antibióticos, representando oito classes diferentes, por difusão em placa, segundo os critérios do CLSI (2008). O fenótipo de resistência observado foi o seguinte: 1% (1/75) para a ampicilina, 1% (1/75) para a amoxicilina/ácido clavulâmico, 100% (75/75) para cefalexima, 76% (57/75) para a cefotaxima, 3% (2/75) para o cloranfenicol, 12% (9/78) para a eritromicina, 71% (53/75) para a gentamicina-10, 0% (0/75) para a gentamicina-120, 100% (75/75) para o ácido nalidixico, 36% (27/75) para a penicilina G, 100% (75/75) para a estreptomicina, 100% (75/75) para o sulfametoxazol/trimetropim, 60% (45/75) para a tetraciclina e 20% (15/75) para a vancomicina.

Seguindo as recomendações do CLSI (2008), para as estirpes de enterococos resistentes à vancomicina, foi ainda avaliada a concentração mínima inibitória (MIC), tendo cinco das estirpes sido classificadas como resistentes a elevados níveis de deste antibiótico (MIC>256 μg/ml).

Para os isolados em que foram observadas resistências nos testes de difusão em placa efectuou-se ainda a pesquisa de genes de resistência por PCR: para a tetraciclina o gene *tet(M)*, para a eritromicina o gene *erm(B)* e para a vancomicina os genes *vanA* e *vanB*. Nas estirpes resistentes à eritromicina 78% foi positiva para a presença do gene *erm(B)*; nas estirpes resistentes à tetraciclina, 65% foi positivo para a presença do gene *tet(M)*; e nas estirpes vancomicina resistentes, nenhuma foi positiva para o gene *vanA* e apenas uma foi positiva para o gene *vanB*.

Tendo em conta estes resultados, todos os enterococos de enchidos tradicionais Portugueses foram classificados como multi-resistentes, ou seja, todos apresentam resistência a pelo menos um agente de três ou mais classes de antibióticos (Magiorakos *et al.*, 2011). Estes elevados níveis de resistência observados são motivo de preocupação, especialmente os elevados níveis de resistência à vancomicina, um medicamento de último recurso no tratamento de infecções potencialmente fatais provocadas por enterococos.

As estirpes de enterococos isoladas de carnes foram ainda avaliadas quanto à presença de genes que codificam factores de virulência, por PCR. Nomeadamente, substancia de agregação (agg), proteína de superfície (enterococcal surface protein –*esp*), adesinas (adhesin-like *E. faecalis* endocarditis antigen - $efaA_{fs}$), pili (endocarditis/biofilm-associated pilus –*ebpABC*), polissacarídeo de parede (enterococcal polysaccharide antigen gene –*epa*), citolisina/hemolisina (*cyl*) e a enzima hidrolítica gelatinase (*gelE*). Foram ainda efectuados testes em placa para pesquisa da actividade hemolítica e da gelatinase.

Os resultados observados para os PCRs de virulência foram os seguintes 5% (4/75) das estirpes positivas para o gene *ebpA,* 19% (14/75) para o gene *ebpB*, 28% (21/75) para o gene *ebpC*, 17% (13/75) para o gene $efaA_{fs}$, 28% (21/75) para o gene *epa*, 25% (19/75) para o gene *fsrB* (parte do operão da gelatinase) e 75% (56/75) para o gene *gelE*. Os genes *agg*, *esp* e *cylA* não foram encontrados em nenhuma das estirpes em análise. Em relação aos testes em placa, 10 estirpes foram classificadas como produtoras de gelatinase (13%) e nenhuma foi considerada β-hemolitica.

As estirpes de enterococos foram também testadas quanto à sua capacidade de produção de biofilmes, tendo sido classificadas em quatro classes diferentes: 8% como não produtoras (6/75); 28% como fracas produtoras (21/75); 25% como produtoras moderadas (19/75); e 39% como fortes produtoras (29/75).

Finalmente, as estirpes de enterococos foram ainda avaliadas quanto ao seu potencial tecnológico, mais precisamente quanto à capacidade de produção de bacteriocinas e actividades enzimáticas (e.g. fosfatase ácida, fosfatase alcalina, entre outras). Apenas 8% (5/75) das estirpes apresentaram

potencial bactericinogénico. Após amplificação por PCR apenas foi possível identificar os genes estruturais das bacteriocinas em duas destas estirpes, correspondendo à enterocina A e enterocina B.
Algumas estirpes apresentaram potencial tecnológico, uma vez que têm capacidade de produção de bacteriocinas e largo espectro enzimático. Contudo, o tipo de aborgadem utilizada neste estudo permitiu perceber que não são passeiveis de serem utilizadas em tecnologia alimentar, uma vez que também apresentam variadas resistências a antibióticos bem como alguns factores de virulência. Uma forma de ultrapassar este facto seria a utilização de extractos purificados de bacteriocinas bem como de enzimas.
Em conclusão, apesar do enterococos isolados de produtos cárneos tradicionais Portugueses apresentarem várias resistências a antibióticos e capacidade de formação de biofilmes, uma vez que estão associados a uma baixa frequência de factores de virulência, um baixo risco parece estar associado ao consumo destes produtos.

Palavras-chave: Produtos cárneos fermentados, *Enterococcus*, interesse tecnológico, potencial patogénico.

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1. Introduction

1.1. General characterization of the genus *Enterococcus*

The *Enterococcus* genus is a very heterogeneous group of gram-positive lactic acid bacteria, both at phylogenetic and phenotypic levels. This group has a very controversial taxonomy that evolved vastly over the decades. Their phenotypic flexibility and resilience allows them to be ubiquitous.

The genus *Enterococcus* consists of gram-positive, facultative anaerobic microorganisms with and ovoid shape that may be organized in short chains, in pairs, or as single cells. Like streptococci, these organisms do not have cytochrome enzymes and are thus catalase negative, although some strains do produce pseudocatalase (Murray, 1990).

The first description of enterococci occurred in 1899 when Thiercelin observed what he first called "*entérocoque*" to emphasize the intestinal origin. The genus *Enterococcus* was proposed by Thiercelin and Jouhaud (1903) for gram-positive diplococci of intestinal origin. Latter, in 1906 Andrews and Horder suggested the name *Streptococcus faecalis* for an *enterococci*-like microorganism isolated from a patient with endocarditis (Stiles and Holzapfel, 1997; Klein, 2003).

In 1933 Lancefield described serological groups for streptococci in which those of 'fecal origin' possessed the group D antigen (Franz *et al.*, 1999; Klein, 2003). The enterococci were described by Sherman (1937) as microorganisms that grow at 10 and 45°C, in 6.5% NaCl and at pH 9.6, survive heating at 60°C for 30 minutes and react with Lancefield group D antiserum, but not all the species share all these characteristics (Murray, 1990; Stiles and Holzapfel, 1997). He also divided the streptococci into four groups: the so called 'enterococci' (or fecal streptococci), the dairy streptococci 'lactic', the 'viridans' group and the 'pyogenous' streptococci (Klein, 2003).

In 1984-1987, based in molecular characteristics such as DNA-rRNA hybridization, the genus *Streptococcus sensu lato* was divided by Schleifer and Kilpper- Bälz, into *Streptococcus sensu stricto*, the genus *Enterococcus* and the genus *Lactococcus*. The differences can be demonstrated by 16S rRNA sequence comparisons, DNA:DNA and DNA:rRNA hybridization as can be observed by analyzing the rRNA 16S phylogenetic tree presented in Figure 1, where *Enterococcus*, *Streptococcus* and *Lactococcus* are divided into different subgroups (Devriese *et al.*, 1993; Franz *et al.*, 1999; Klein, 2003).

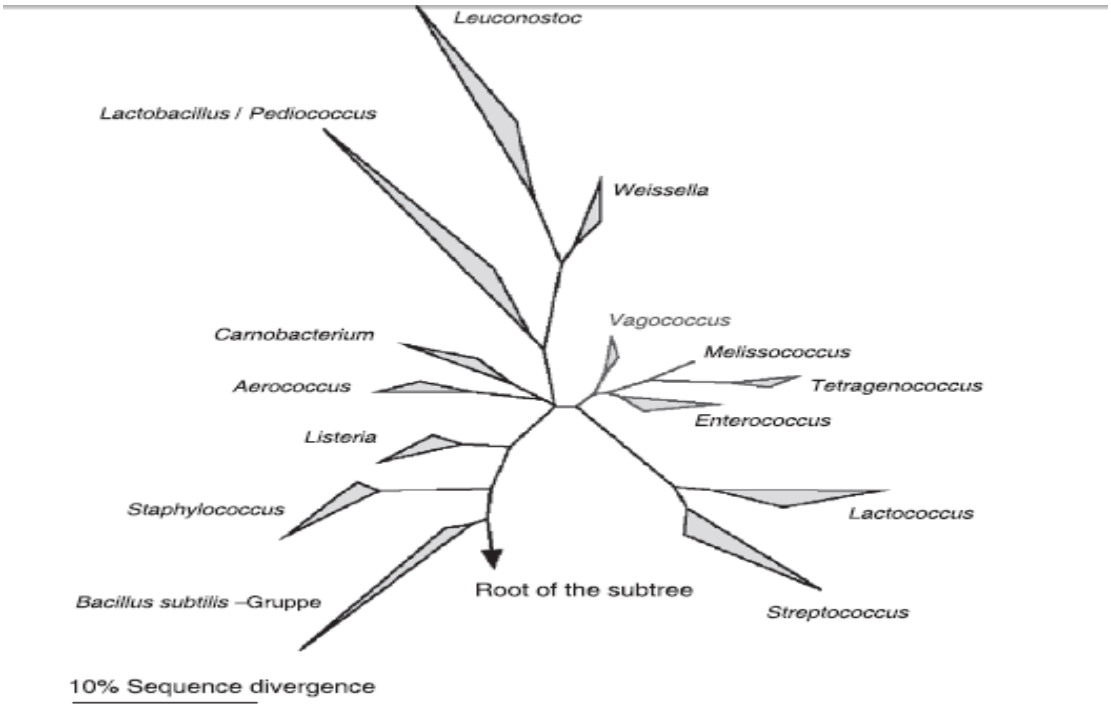

Figure 1 - The phylogenetic position of the genus Enterococcus demonstrated by a 16S rRNA-dendrogram of Gram-positive genera (Klein, 2003).

The most recently described species have considerable differences in their physiological and biochemical behavior compared to the typical enterococci (Klein, 2003). According to the Taxonomic Outline of *Bacteria* and *Archaea* 7.7 (TOBA), in 2007 there were 33 species identified of enterococci. According to J. P. Euzéby [http://www.bacterio.cict.fr/e/enterococcus.html Date of consultation 10/09/2011] there are currently more than 30 different species cited in published scientific articles. In 2011 two new species were described, *Enterococcus ureasiticus* sp. nov. and *Enterococcus quebecensis* sp. nov. (Sistek *et al.*, 2011).

Enterococci are chemoorganotrophic and produce L-lactic acid from hexoses by homofermentative lactic acid fermentation (Franz *et al.*, 2003). The fermentation occurs in anaerobic conditions, in which these bacteria metabolize some sugars (as glucose) and use organic molecules as their final electron acceptor to produce end products (lactate).

As mentioned before, enterococci can grow from 5-10ºC to 45-50ºC, in aerobic and anaerobic environments. Both *E. faecium* and *E. faecalis* can survive heating at 60 ºC for 30 minutes, as well as a range of pH values between 4.6 and 9.9. Enterococci can tolerate the presence of bile salts up to 40% (w/v). *E. faecalis* is able to grow in 6.5% of NaCl.

However, at present there are no phenotypic criteria available which unequivocally separate the genus *Enterococcus* from the other genera of Gram-positive, catalase-negative cocci (Devriese *et al.*, 1993), as seen in Table 1 which presents a brief summary of the phenotypic characteristics usually attributed to enterococci. It can be observed that there is no consistency in the genus concerning phenotypic features, hence the growing importance of molecular-based identification schemes.

Table 1 - Characteristic physiological properties of some enterococcal species.

Species	Growth conditions					Esculin hydrolysis	Group D antigen
	10ºC	45ºC	pH 9.6	6.5% NaCl	40% Bile		
E. asini	(+)	(+)	n.d	-	+	+	+
E. avium	V	+	+	V	V/+	+	+
E. casseliflavus	+	+	+	V/+	+	+	+
E. cecorum	-	+	(+)	-	(+)	+	-
E. columbae	-	n.d	n.d	-	(+)	+	
E. díspar	+	-	n.d	+/-	+	+	-
E. durans	+	+	+	+	+	+	+
E. faecalis	+	+	+	+	+	+	+
E. faecium	+	+	+	+	+	+	V
E. flavescens	V/-	V/+	n.d	+	+	+	+
E. gallinarum	+	+	+	+	+	+	+
E. haemoperoxidus	+	-	n.d	+	+	+	+
E. hirae	+	+	+	+	+	+	V
E. malodoratus	+	-	+	+	+	+	+
E. moraviensis	+	-	n.d	+	+	+	+
E. mundtii	+	+	+	+	+	+	+
E. porcinus	+	+	n.d	+	n.d	+	+
E. pseudoavium	+	+	+	+/-	V/+	+	-
E. raffinosus	(+)	+	+	+	V/+	+	n.d
E. ratti	+	+	n.d	+	n.d	+	(+)
E. saccharolyticus	+	+	n.d	(+)	+	+	-
E. sulfureus	+	-	n.d	+	+	+	-
E. villorum	n.d	n.d	n.d	+	+	+	n.d

Legend: n.d., not determined; (+), weak positive; V, variable; +/-, differing reports in literature (Adapted from Franz *et al.*, 2003, and Devriese *et al.*, 1993).

On the other hand, it should be noticed that positive reaction to Lancefield group D antiserum is not exclusive to enterococci, as species of *Streptococcus* spp. give positive reactions. Other example is growth at 6.5% NaCl, which is also observed for lactococci, pediococci, aerococci and leuconostocs; pediococci and some lactococci are able to grow at 45 ºC, while lactococci, leuconostocs and some streptococci usually grow at 10°C. Another test that, so far, has proven to be positive to all species, is the hydrolysis of esculin as well as the tolerance to 40% (v/v) bile. However, *Streptococcus bovis*, some pediococci and most lactococci, leuconostocs and aerococci also react positively. Testing the hydrolysis of different carbon sources would have similar results, meaning that the different species would have different behaviors for the same carbon source (Devriese *et al.*, 1993).

Since the identification solely based on phenotypic traits fails to discriminate several species, nowadays identification is usually obtained resorting to molecular methods such as typification (Ŝevc *et al.*, 2005), sequencing of 16S rRNA (Patel *et al.*, 1998), PCR-amplification of the *tuf* gene (Ke *et al.*, 1999), ITS-PCR and subsequent restriction analysis (ARDRA) (Alves *et al.*, 2004), SDS-PAGE of whole-cell proteins (Alves *et al.*, 2004), among others.

As we have seen the *Enterococcus* genus is a phenotypically heterogeneous group and there is a growing need for molecular methods that allow proper identification of both genus and species. The

phenotypical approach for the identification should be used as presumptive and as a complementary method.

1.2. Environmental Sources and Reservoirs

The *Enterococcus* genus comprises a very diverse and complex group of ubiquitous bacterial, usually found in the gut of humans and other warm blooded animals, in soils, water, plants and even in several foods. They ubiquitous nature is related to their phenotypic flexibility and resilience.

In general, enterococci are a part of the autochonous microbiota present in the gastrointestinal tract of humans and other animals, with some species being more commonly associated with certain animals. *E. faecalis and E. faecium* are the most frequent species found in human intestines, as well as in fowl intestines.

Enterococci are also frequently found in food, mainly *E. faecalis and E. faecium*, with *E. faecalis* being more frequent in food and feces from animal origin than *E. faecium*. There are various reports but, according to Franz *et al.* (1999), the numbers of *E. faecalis* in human feces range from 10^5 to 10^7 CFU/g, and from 10^4 to 10^5 CFU/g for *E. faecium*. In production animals like cattle, pigs and poultry *E. faecium* is a frequent species, followed by *E. faecalis* and *E. cecorum*. Less frequent are *E. gallinarum* and *E. durans/hirae* or *E. avium*.

Some enterococci have detrimental activities that include spoilage, especially in meats; the majority is heat tolerant and/or freezing resistant and can resist food processing. However, being LAB, they play an important role thanks to their fermenting activities. This unique character makes them responsible for the development of sensory characteristics of some cheeses and sausages (especially in the Mediterranean area), resulting in products with special organoleptic attributes that make them “delicacies”. They are also associated with the natural fermentation in black olives (Bevilacqua *et al.*, 2010).

As natural inhabitants of the intestines, enterococci presence has been used as an indication of fecal contamination for both water and food (Klein, 2003). Hence, there are a number of reports in these sources.

Overall, enterococci can be found in a variety of sources; a summary of the reported enterococcal ecological niches can be seen in Figure 2.

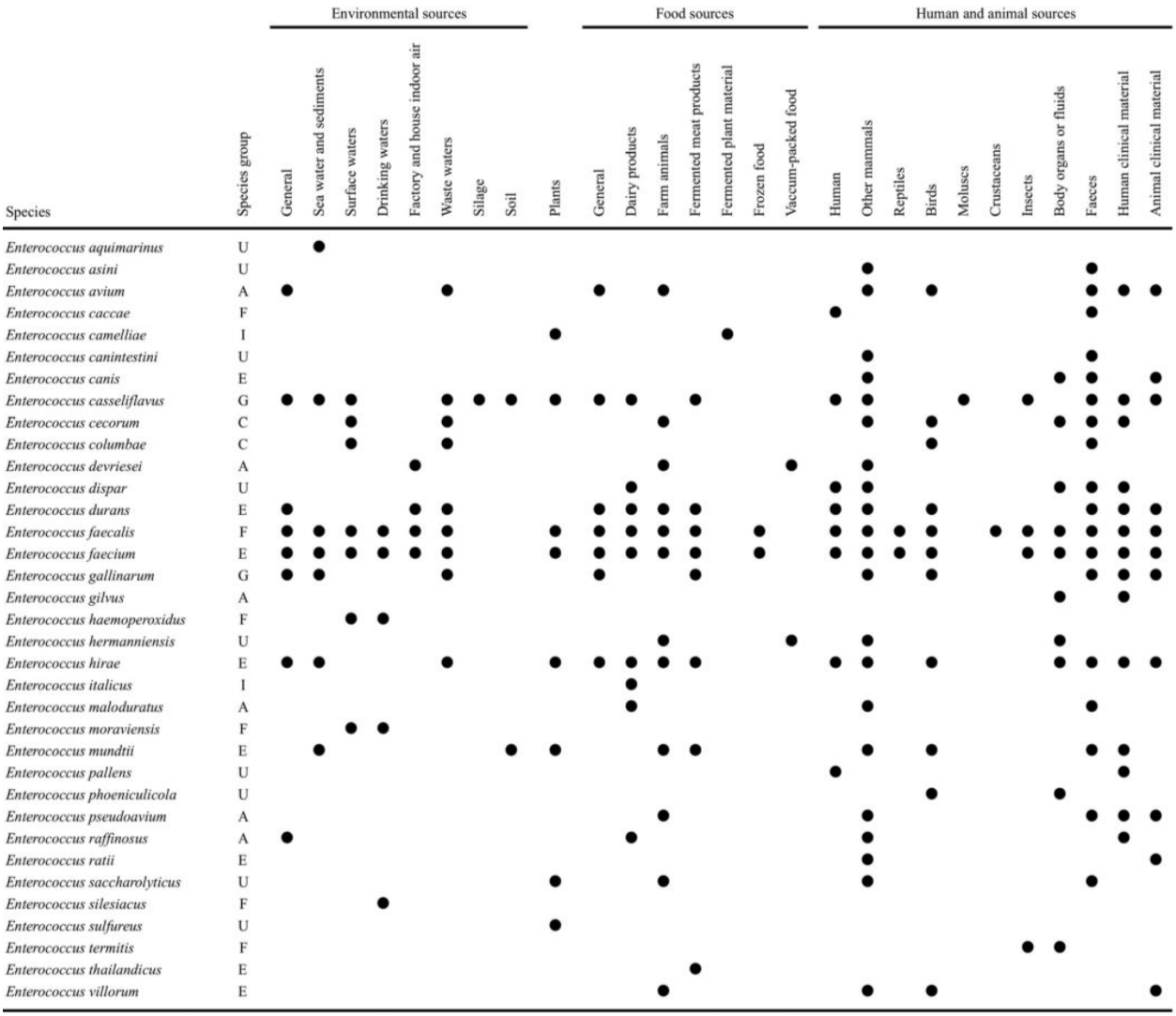

Figure 2 - Main known ecological niches of the currently described 35 species of the genus *Enterococcus* (Lemsaddek and Tenreiro, 2011**).**

1.2.1. Enterococci in meat products

Enterococci have a major role in food production, since they are frequently found in foods, are very resilient and posses phenotypical characteristics beneficial to the end product organoleptic traits.

Since enterococci are present in the gastrointestinal tract of animals its presence in meat is usually associated with contamination at the time of slaughtering. Pig carcasses can contain from 10^4 to 10^8 CFU/100 cm^2, with *E. faecium* and *E. faecalis* being the most frequently found.

In Mediterranean countries, such as Portugal, many meat fermented products are manufactured carrying out the artisanal ways. The low-acidity of the products and the fermentation carried out without the use of starter cultures allow the natural microbiota to be constituted mostly by LAB, including enterococci. In addition, the persistence of enterococci during ripening can be attributed to their wide range of growth temperatures and their high tolerance to salt (Hugas *et al.*, 2003). Enterococci can be conveyed to meat and meat products by two main routes, from the slaughtering process or as part of the fermentative microbiota (Crespo and Alves, 2011).

Enterococci are among the most thermotolerant of the non-sporulating bacteria, posing a problem in processed meat products, which are not always cooked, being typically salty, cured, or raw. On the other

hand, cooking of processed meat products usually undergoes at 60ºC to 70ºC, temperatures to which enterococci can survive (Franz *et al.*, 1999). During fermentation, the contaminating enterococci, related with intestinal or environmental colonization, may survive and multiply, especially if the product has no competitive starter culture. The contamination may also be a cross-contamination in the final stages of food processing such as slicing, packing or handling.

Hugas *et al.* (2003) and Klein (2003) summarized a series of isolations of enterococci from meat products (Table 2). They found reports from contaminated poultry, pork and beef (10^2-10^4 CFU/g), Greek natural fermented sausages (10^2-10^3 CFU/g), German and Italian fermented sausages (10^3-10^5 CFU/g), Spanish naturally fermented sausages (1.3 to 4.48 log CFU/g). Both Barbosa *et al.* (2009) and Ribeiro *et al.* (2011) reported the presence of enterococci in traditional Portuguese fermented meat products.

The exact role of enterococci in meat fermented products has not been thoroughly studied. In meat fermented products, the dominant microbiota is constituted by several species LAB, mainly of the genus *Lactobacillus*. *Enterococcus* (*E. faecalis*, but especially *E. faecium*), represent some of the LAB species that can be found in relatively high numbers during meat fermentation. It's thought they may contribute, together with lactobacilli, to the fermentation process (Hugas *et al.*, 2003), but the biochemical activities of enterococci in sausages are yet to be studied. They might contribute to sausage aromatization by their glycolytic, proteolytic and lipolytic activities (Giraffa, 2002).

Table 2 - Occurrence of enterococci in different food products (Klein, 2003).

				Sausages				
Species	**Cheese**	**Meat**	**Pork carcasses**	**Fresh**	**Expired**	**Spoiled**	**Minced beef**	**Minced pork**
E. faecalis	(+)	+	++	++	+	(+)	++	++
E. faecium	++	++	(+)	-	-	++	(+)	(+)
E. durans/hirae	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
E. gallinarum	-	(+)	n.d	n.d	n.d	n.d	(+)	(+)
E. casseliflavus	-	(+)	n.d	n.d	n.d	n.d	-	(+)
E. mundtii	-	(+)	n.d	n.d	n.d	n.d	n.d	n.d
E. avium	n.d	n.d	n.d	n.d	n.d	n.d	-	(+)
E. malodoratus	n.d	n.d	(+)	(+)	(+)	-	n.d	n.d
E. pseudoavium	n.d	n.d	(+)	-	+	-	n.d	n.d
E. raffinosus	n.d	n.d	(+)	-	(+)	-	n.d	n.d

Legend: ++: usual; +: frequent; (+): occasional; -: not mentioned; n.d: not investigated.

The present investigation studied several Portuguese traditional fermented meat products namely, Catalão, Chouriço Preto, Linguiça, Paio and Salsichão.

Catalão is basically a mixture of Alentejano/Ibérico pork, wine, peper and cumin. Chouriço Preto is a mixture of Alentejano/Ibérico pork, blood, and mass of pepper, ground garlic, black pepper, cumin and cloves. Linguiça is a mixture of Alentejano/Ibérico pork, wine, mass of pepper, ground garlic, salt and bay. The casings of these three products are natural clean bowel, and the packing had a protective atmosphere (gas, composition unknown).

Salsichão is a mixture of Alentejano/Ibérico pork, wine, peper and cumin. Paio is constituted basically by a mixture of Alentejano/Ibérico pork, wine, mass of pepper, ground garlic, salt and bay. The casing of both products is a natural clean bowel, and the packing had vacuum atmosphere.

Meat fermentation has been used for centuries to extend the preservation of raw materials. It improves both safety, due to the inactivation of pathogens, and stability, by extending inhibition of undesirable changes brought about by spoilage microorganisms. The biochemical and physical modifications that occur during the ripening period change the organoleptic characteristics of the raw material and generate end products, which possess completely distinct organoleptic properties. Moreover, meat fermentations lead to an enormous variety of products, which are much appreciated by consumers all over the world. In meat the enterococcal functional properties have not been studied in the same detail nevertheless, enterococci are considered to contribute to the final flavor of the product due to glycolytic, proteolytic and lipolytic activities (Crespo and Alves, 2011).

1.2.2. Enterococci in dairy products

Enterococci constitute a major component of the microbiota of artisanal cheeses produced in southern Europe, including Portugal, and play an important role in ripening and aroma development (Eaton and Gasson, 2001). The cheese enterococci originate from the different types of milk used to produce cheeses, from ewe's, goat's, water-buffalo's and cow's milk, either raw or pasteurized (Crespo and Alves, 2011).

In contrast to meat products, the benefits of certain *Enterococcus* have been largely reported in cheeses (Franz *et al.*, 1999). This has led to suggestions for the inclusion of enterococci in starter culture preparations for the manufacture of certain Mediterranean cheeses (Eaton and Gasson, 2001).

Enterococci are frequently associated with traditional fermented cheeses. The presence of enterococci in milk can be through feces, contaminated water or even from contaminated materials. In cheeses enterococcal frequency can range from 10^4 to 10^6 CFU/g in cheese curds, and from 10^5 to 10^7 CFU/g in fully ripened cheeses (Franz *et al.*, 1999).

There are several reports of cheese products which involved enterococci such as Comté and Saint Nectaire cheeses (France); traditional goat and buffalo cheeses, artisanal Fiore Sardo, traditional Alps, artisanal Montasio and goat cheeses (Italy); model curdled goat cheeses, ewe's milk and cheeses from Portuguese Registered Designation of Origin Areas; feta cheeses (Greece); Cebreiro raw cow's milk cheeses and Serra ewe's cheeses (Spain), Tolminc cheeses (Slovenia); and Chedar cheess (Ireland) (Ogier and Serror, 2008).

Enterococci are applied in food industry in fermentation processes or for the upgrading of some sensorial traits of food. These bacteria may play a beneficial role in the production of various fermented food products and may also be added to food as probiotics. The presence in fermented foods such as cheeses results in organoleptical features unique to traditional production, due to their proteolytic and estereolytic activities as well as the production of diacetyl and acetoin (Franz *et al.* 1999; Giraffa, 2002). Another beneficial role of enterococci is the production of bacteriocins with large antimicrobial spectrum against pathogenic bacteria (Franz *et al.*, 2007).

1.3. Food technological potential

The technological potential of enterococci is related to their low acidification ability, proteolytic and lipolytic activities, carbohydrates metabolism, production of volatile compounds and bacteriocins. The evaluation

of the presence of these traits allows the initial selection of enterococcal strains to be uses in starter cultures in food fermentations, or even as probiotics.
Also related to the technological potential are the phenotypical plasticity features of enterococci, reflected in their ability to withstand heat and freezing temperatures, acidity and a wide variety of salt concentrations.

1.3.1. Enzymatic activities

The presence of enterococci in high numbers in some fermented foods is linked with their biochemical activities that eventually contribute to the sensorial traits of those products.
Enterococci produce L-lactic acid along from hexoses by means of homofermentative lactic acid fermentation. Although the main product is lactate, they can also produce significant amounts of acetate, formate and ethanol, depending on growth conditions. All of said compounds are important in determining the taste of many fermented products (Giménez-Pereira, 2005).
The ability to produce side products is associated with the presence of different enzymes. These enzymes allow the conversion of different carbon sources typical for each staring material, eater milk or meat, to be converted. There are reports involving enterococci in the production of formic and acetic acid, and even citric acid.
Typically associated with enterococci is the ability to hydrolyze milk casein (important in cheeses production) and peptidolytic, esterase and lipase activities (important in dairy and meat products). Those activities are all important to both flavor and texture (Giménez-Pereira, 2005; Crespo and Alves, 2011).
The breakdown of lactose and citrate during cheese ripening can give rise to a series of volatile compounds like acetaldehyde, ethanol and acetoin, which may further contribute to flavor development of fermented dairy products.
The presence of esterase, esterase-lipase, α-and β-glucosidade, β-galactosidase and N-acetyl-β-glucosaminidase activities has already been reported for both *E. faecalis* and *E. faecium* (Crespo and Alves, 2011).
All these traits seem to be strain-specific. Considering these features, enterococci can be used as starter cultures with the purpose of improving the overall sensory properties of distinct fermented food products.

1.3.2. Bacteriocinogenic properties

Bacteriocins are ribosomally synthesized peptides with bactericidal activity directed against species that are usually closely related to the producer bacterium.
The application of bacteriocins in food biocontrol is mainly oriented in two alternative directions, the use of bacteriocin-producing bacteria or the direct addition of bacteriocin preparations, from the supernatant culture of the producer strains.
The *in situ* production of bacteriocins may increase the competitiveness of the producer strain in the food matrix and contribute to the prevention of spoilage. However, the endogenous microbiota, may influence the performance of the bacteriocinogenic cultures by inadequate environment for growth and/or bacteriocin production, loss of bacteriocin-production ability, antagonism by other microflora, development of bacteriocin-resistant organisms and formation of non-active complexes between bacteriocins and macromolecules (Giraffa, 2002). The use of bacteriocin preparations also eliminates the risk associated with the producer enterococcal strain virulence.

The antimicrobial activities of the lactic acid bacteria have been known for a long time and recognized as important in food fermentations, food preservation and intestinal ecology, especially concerning some food-borne pathogens like *Listeria monocytogenes* and *Staphylococcus aureus* (Klaenhammer, 1993; Nes *et al.*, 1996).

The bacteriocin genes encode a pre-form of the bacteriocin containing an N-terminal leader sequence (called the double-glycine leader) whose function seems to be preventing the bacteriocin from being biologically active while inside the producer, and to provide the recognition signal for the transporter system (Klaenhammer, 1993). The mature bacteriocins identified so far vary in size from less than 30 residues to more than 100 residues in some cases.

Bacteriocin producers have developed a protection system against their own bacteriocin, referred to as immunity. Each bacteriocin has its corresponding small protein (51-150 amino acids) conferring immunity, which is expressed concomitantly with the bacteriocin, but since these immunity proteins and bacteriocin share no similarity, it appears that there is no direct interaction between the two.

It has been occasionally observed that bacteriocinogenic LAB can lose their ability to produce bacteriocins. Some of these observations have been attributed to plasmid loss or to transposition-mediated inactivation. Recently, it has been shown that production of some bacteriocins can be transcriptionally regulated and that bacteriocins can not be produced in the absence of an induction factor (IF). This phenomenon may also explain why loss of bacteriocin production is occasionally observed.

The production of bacteriocins is self-inducible, but the way this system is activated is not clear. There are two models to explain this. One assumes that the induction factors are constitutively produced in small quantities; hence the induction would depend on cell density. The other model suggest that the constitutive production of the inducer factor is kept at concentration just below activation and that changes in the environment, such as changes in nutrients, among others, would cause a short and temporary increase in the production of the IF (Nes *et al.*, 1996).

Enterococci are one of the most common bacteriocin-producing LAB. For the specific case of enterococcal-bacteriocins they are generically named enterocins. The most common enterocins are produced by *E. faecalis*, *E. faecium* and *E. mundtii* and include enterocins A, P, 1071A and B, mundticin, bacteriocin 31, among others (Franz *et al.*, 2007).

Franz *et al.* (2007) proposed a classification scheme for enterocins, with four classes: Class I enterocins (lantibiotic enterocins), Class II enterocins (small, nonlantibiotic peptides), Class III enterocins (cyclic enterocins) and Class IV enterocins (large proteins). Class II can be subdivided into three subclasses: II.1, enterocins of the pediocin family; II.2, enterocins synthesized without a leader peptide; and II.3, other linear, nonpediocin-type enterocins. Cytolysin produced by *E. faecalis* is the only lantibiotic-type enterocin currently known (Franz *et al.*, 2007).

One example of a Class I enterocin is cytolysin, a two-peptide bacteriocin with both structural subunits containing lanthionine residues.

In Class II, the pediocin-like bacteriocins, all contain a hydrophilic cationic region with a pediocin box motif (YGNGVXC), and two cysteine residues joined by an S–S bridge stabilizing the formed b-sheet structure. This group includes enterocin A, P, L50AB, Q, 1071AB and B.

Class III includes the bacteriocins that are ribosomally synthesized, post-translationally unmodified and head-to-tail linked, cyclic antibacterial peptides. An example is enterocin AS-48 from *E. faecalis*.

Class IV includes the enterocins, hence produced by enterococci. Enterolysin A is an example; it is a large, heat-labile bacteriocin (Franz *et al.*, 2007).

As seen before, the nature of the bacteriocins can be exceptionally diverse. Some can be encoded in conjugative or pheromone-responsive plasmids. The genetic transfer mechanisms, like conjugation, may explain the variety of enterocins and the fact that multiple bacteriocins can be produced by a single strain; and that the same enterocin can be produced by different strains. On the other hand antibiotic resistance genes can also be present in these elements. In this way bacteriocin production can contribute as a competitive advantage for enterococci, especially allied with antibiotic resistance may enable the enterococci to establish themselves among a diverse microbiota, colonize and infect (Franz *et al.*, 2007).

Bacteriocin production may also play an important role in food colonization, especially in dairy or meat fermented products. However, it should be noted that enterococcal success is not only due to the production of bacteriocins but is combined with other factors, such as tolerance to adverse conditions and enzyme production (Franz *et al.*, 2007).

Enterocin can have a broad activity spectrum against other LAB, but they are especially active against *Listeria monocytogenes*. This is important considering that enterococci are a major component of the microbiota of certain cheeses and other dairy products where *L. monocytogenes* is frequently a spoilage microorganism. To date there is only one bacteriocin licensed for use as a food additive, a Class I, lantibiotic-type, nisin. Other bacteriocins are naturally present in foods through production by starter cultures or even by bacteria naturally present in the production process (Franz *et al.*, 2007).

1.4. Pathogenicity potential

Enterococci have a pathogenicity potential associated, i.e. they have the ability to cause illness but do not necessarily do so. These bacteria are traditionally viewed as commensal inhabitant of the gastrointestinal tract of humans and animals but are also recognized as potential human/animal pathogens.

Even though enterococci are widely spread in foods, to date, there is no report of an infection caused by enterococci after the consumption of a contaminated food product.

What seem to influence the difference between a potential and an effective pathogen is the presence/expressionof virulence factors in combination with antibiotic resistances.

Unlike most lactic acid bacteria, the *Enterococcus* genus is not considered “generally recognized as safe” (GRAS). Even though enterococci have been considered useful in cheese and fermented meat production, there is controversy due to the fact that, over the last decades enterococci have emerged as opportunistic pathogens for humans (Ogier and Serror, 2008).

The first probable report of a human disease associated with enterococci was in 1912. Although the definition of the *Enterococcus* genus was not exactly the same as today, the symptoms described are consistent with an enterococcal infection (Murray, 1990).

Enterococci have been established as major nosocomial infectious agents since the 1970s and 1980s (Jett *et al.*, 1994). They are considered as opportunist pathogens, i.e., harmless for healthy individuals that can become pathogenic mainly towards hospitalized patients with severe underlying diseases, compromised immune systems, children or elderly people. Enterococci are frequently associated with other pathogens in polymicrobial infections (Ogier and Serror, 2008).

According to the 2010 Annual Epidemiological Report on Communicable Diseases in Europe (ECDC – European Center of Control Disease), approximately 80% of human clinical enterococcal infections are caused by *E. faecalis,* and for this species high-level aminoglycoside resistance is of special concern. The remaining 20% being caused mainly by *E. faecium,* for which resistance to vancomycin is of special concern. Although the number of vancomycin-resistant *E. faecium* isolates reported to European Antimicrobial Resistance Surveillance System (EARSS) in 2008 was low, and the occurrence of vancomycin resistant enterococci (VRE) was less than or equal to 5%, or even absent, in 13 of the 24 countries that reported at least ten *E. faecium* isolates. By contrast, three countries (Greece, Ireland, and the UK) reported more than 25% VRE isolates. Increasing trends in the occurrence of VRE were observed only in Slovenia. Decreasing trends were observed for France, Greece and Italy. According to this same report, in the year 2008 in Portugal, 0.03% (total of 391) of the clinical isolates in intensive care unit-acquired pneumonia were *Enterococcus* spp., being the tenth most frequent; and 11.5% (total of 209) of the clinical isolates in ICU-acquired bloodstream infections were *Enterococcus* spp., being the second most frequent after coagulase-negative staphylococci.

The most common human infections, caused by enterococci, are in the urinary tract, followed by bloodstream, endocardium, abdomen, biliary tract, burn wound and indwelling foreign devices, such as catheters. Less frequently enterococci infect lungs, soft tissues, paranasal sinuses, ears, eyes and periodontal tissue (Semedo-Lemsaddek and Mato, 2011).

Enterococci have genetic movable elements able to pass from strain to strain via conjugation. Some plasmids are specific to enterococci while others have a broader range within gram-positive bacteria. Enterococci also have conjugative transposons that are able to transfer then self without plasmid mobilization (Clewell, 1990). The presence of some virulence traits and antibiotic resistance traits in movable elements increases the risk associated with the presence of virulence factors in food-enterococci.

1.4.1. Virulence factors

Despite the fact that enterococci are a health care problem worldwide and are widespread in a variety of environments, the mechanisms of enterococcal pathogenesis are not yet well understood. About a dozen putative virulence factors have been reported for enterococci, especially *E. faecalis*, from adhesins to secreted factors.

As mentioned before, enterococci can cause several human infections. The fundamental steps of an infectious disease start with an environmental persistence, since enterococci are resilient and can colonize a variety of niches. Next is the attachment and entry into the human body; followed by a local or general spread, which includes tissue damage. This provokes the host's defenses which the pathogen (enterococci) must then evade (hydrolysis of immune system components and capsules). The final step is causing disease, due to a variety of virulence factors (Semedo-Lemsaddel and Mato, 2011).

Next, follows a brief list of the most common enterococcal virulence factors.

<u>Adhesins</u>

Enterococci produce adherence substances that allow specific means of attachment, without which they would likely be eliminated by content flow through normal intestinal motility. Through these surface-exposed adhesins the bacterial cells can adhere to epithelial cells, endothelial cells, leukocytes, or extracellular matrix, among other, in which is generally the first step in infection (Jett *et al.*, 1994).

Aggregation substance (AS) is encoded in sex-pheromone plasmids like pAD1 or in chromosomal pathogenicity islands (Semedo-Lemsaddk and Mato, 2011). AS is a surface-bound protein encoded by pheromone-responsive plasmids of *E. faecalis* and expressed in response to pheromone induction. Aggregation substance promotes aggregation or clumping between plasmid positive donor cells and pheromone-producing recipient cells and facilitates transfer of plasmids during bacterial conjugation (Dunny, 1990).

AS contributes to virulence both by promoting plasmid encoded virulence factors and antibiotic resistance dissemination and by facilitating colonization through the promotion of adhesion to extracellular matrix and other proteins, host cells and also to plastic polymers and invasion of cells and tissues during infection (Semedo-Lemsaddek and Mato, 2011).

The *Enterococcus faecalis* antigen A (*efaAfs*) was first described by Lowe *et al.* (1995), in the serum from a patient with endocarditis. In 1998, Singh *et al.* disrupted the *efaAfs* gene and used the *efaAfs* mutant in an *in vivo* mice test and observed a more prolonged survival, proving its influence in pathogenicity.

Another example of adhesin is the enterococcal surface protein (Esp) that has been reported as being involved in binding to abiotic surfaces, components of the extracellular matrix and eukaryotic cells and to be implicated in biofilm formation. However, contradictory theories have been published over the last years about the role of *esp* and its specific role has not been defined so far.

A homologue has been identified by Eaton and Gasson (2001) for *E. faecium*; Esp of *E. faecium* shares up to 90% homology with Esp of *E. faecalis*, but its specific function is currently unknown (Heikens *et al.*, 2007).

Toledo-Arana *et al.*, (2001) defined the Esp structure, as a large surface protein composed of 1873 amino acids with an N-terminal domain of 50 to 743, that has no similarity to other proteins in the database. The central core has 744 to 1665 amino acids and has global structural similarity to C alpha and Rib proteins of group B streptococci. The C- terminal domain of 1666 to 1873 amino acids contains a membrane-spanning hydrophobic region that includes a slight modification of the LPXTGX motif found in most wall-associated surface proteins of gram-positive bacteria. The hypothesis is that the N-terminal region of Esp might interact with the host and that the central region might be used to retract the protein from the surface, hiding it from the immune system (Toledo-Arana *et al.*, 2001).

In this work Toledo-Arana *et al.* (2001) disrupted the *esp* gene in some biofilm producer strains to analyze the possible role of Esp protein in *E. faecalis* biofilm formation. They found some strains in which they disrupted the *esp* gene did not significantly decrease their ability to produce biofilm. This strongly suggests the involvement of other surface proteins that might mediate initial attachment to abiotic surfaces in the absence of *esp*. On the other hand, 87 out of 93 strains harboring the *esp* gene were able to produce biofilm, while none of the *esp* deficient strains were able to produce biofilm. This strongly suggests an involvement of this gene in the biofilm formation process of *E. faecalis*. Other reports support the involvement of *esp* in biofilm formation (Tendolkar *et al.*, 2004), including in *E. faecium* (Heikens *et al.*, 2007). On the contrary, others reported Esp-independent biofilm formation in *E. faecalis* (Kristich *et al.*, 2004).

Also involved in biofilm formation are the *ebp* genes. Nallapareddy *et al.* (2006) reported that all three Ebp (A, B and C) proteins are components of *E. faecalis* surface pili and that mutations in those genes markedly reduced biofilm formation. Mutation in *ebpABC* genes implicate differences in the initial steps of biofilm formation, indicating that *ebpABC* are involved in the early attachment and cell-surface

interactions of biofilm formation. These virulence factors are included in the MSCRAMM family, microbial surface component recognizing adhesive matrix molecules, and are involved in the initial steps of infection, including endocarditis. The *E. faecalis* pili appears to be formed by cross-linking of the three Ebp proteins, but the precise mechanisms of action in endocarditis and biofilm formation in not yet completely understood (Nallapareddy *et al.*, 2006).

Hydrolytic Enzymes

Enterococci secrete virulence factors like gelatinase. Gelatinase is an enzyme able to hydrolyze gelatin and collagen, among other protein substrates (Lopes *et al.*, 2006).

In 2000 Qin *et al.* described the *frs* locus, constituted by *fsrABC* genes upstream of the *gelE* gene in *E. faecalis* OG1RF. They found that the FsrA and FsrC proteins resemble response regulators and sensor transducers of bacterial two-component systems, respectively. They have also demonstrated that the *fsr* locus in *E. faecalis* OG1RF positively regulates the expression of *gelE* and is cell density dependent. The expression of all *fsr* genes (*fsrA*, *fsrB* and *fsrC*) is required for the regulatory functions of the *fsr l*ocus, and have only been detected in *E. faecalis*, *E. faecium, E. durans, E. hirae* and *E. dispar*, but may be widespread among the genus *Enterococcus* (Qin *et al.*, 2001; Lopes *et al.*, 2006). The presence of the *gelE* gene does not imply that strains demonstrate a positive phenotype, since a complete operon is required for gelatinase activity (Eaton and Gasson, 2001).

Other secreted factors

The first virulence factor to be studied in enterococci was hemolysin, by Todd in 1934. Today it is also known as cytolysin. It corresponds to a post-translationally modified protein-toxin that causes a β-hemolytic reaction on certain erythrocytes that also presents bactericidal activity against a broad range of gram-positive bacteria (Haas and Gilmore, 1999).

Cytolysin production by *E. faecalis* is recognized by the development of clearing around colonies on certain blood agar media. This phenotype can be overlooked because sheep erythrocytes, the target cells commonly used in blood agar plates, are not lysed. On the contrary, erythrocytes from rabbits, humans, horses, and cows are lysed by the enterococcal cytolysin (Jett *et al.*, 1994). The hemolysin/cytolysin may contribute to enterococcal virulence through its toxic activity or by disrupting local ecology (Gilmore *et al.*, 1994).

The hemolytic phenotype of cytolysin is more often associated with clinical than environmental enterococci and, based on animal pathogenicity models, it is considered that it may contribute to bacterial virulence (Gilmore *et al.*, 1994; Jett *et al.*, 1994; Haas and Gilmore, 1999).

The genes involved in the hemolytic process are located on highly transmissible pheromone-responsive plasmids, like pAD1 (Gilmore *et al.*, 1994), although they can also be found occasionally in the chromosome (Ike *et al.*, 1992). The cytolysin operon contains five open reading frames, *cylL$_L$, cylL$_S$, cylM, cylB and cylA.* The *cylL$_L$* and *cylL$_S$* products, with 68 and 63 amino acids, respectively, are the precursors. The role of *cylM* is not totally understood, but it is hypothesized that it is involved in the ribosomal modification by the incorporation of cysteine, serine and theorine into the characteristic lanthionine residues of the precursor. After the modification the CylL$_L$ and CylL$_S$ precursors seem to be externalized through CylB (an ATP-binding cassette transporter). Once outside the cell, CylA appears to activate one or both cytolysin precursors (CylLL and/or CylLs) by limited proteolysis (Fig. 3) (Gilmore *et al.*, 1994).

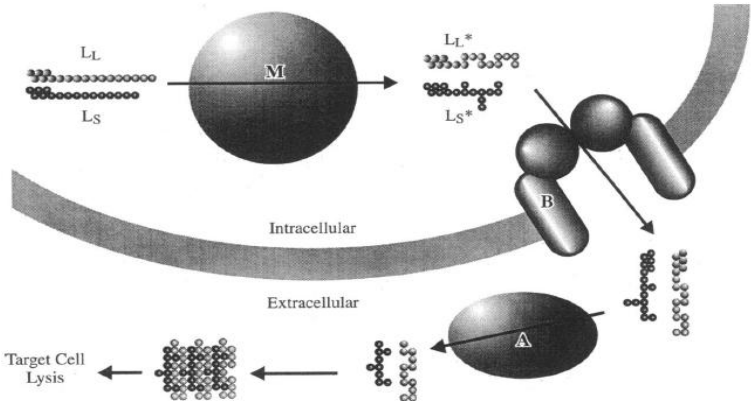

Figure 3 - Model of *E. faecalis* cytolysin maturation, externalization, and activation (Gilmore *et al.*, 1994).

Exopolyscacharides

The enterococcal polysaccharide antigen gene (*epa*), from *E. faecalis* is another virulence factor reported to be involved in enterococcal biofilm formation. Mohamed *et al.* (2004) reported a 73% reduction in biofilm formation in *epa* gene cluster mutants, but was not able to prove the surface location of Epa protein or its direct role in attachment or biofilm accumulation. The *epa* mutant affected primary attachment on polystyrene negatively, but possibly also biofilm accumulation.

The fact that some of the genes mentioned before are sometimes absent in biofilm producing strains, seems to be the reason for the variable capacity of biofilm production, i.e. why some strains are designated as strong producer, other as weak producer and as nonproducers (Nallapareddy *et al.*, 2006).

Some virulence factors are encoded by conjugative plasmids genes, like cytolysin; others are chromosomal like the *fsr* genes or yet encoded in a large chromosomal region described as the pathogenicity island, like *esp* (Ogier and Serror, 2008). Plasmid encoded virulence factors are known to be transmissible by gene transfer mechanisms (Chow *et al.*, 1993; Wirth, 1994).

There have been numerous reports on the distribution of *E. faecalis* and *E. faecium* virulence factors in isolates from different sources, including clinical, commensal, food and environmental isolates (Franz *et al.*, 2001; Mannu *et al.*, 2003; Semedo *et al.*, 2003; Maietti *et al.*, 2007; Valenzuela *et al.*, 2009; Barbosa *et al.*, 2009; Ribeiro *et al.*, 2011). No virulence factor has been characterized in other enterococcal species (Ogier and Serror, 2008) and although there is a higher incidence among clinical strains, virulence traits have also been detected in food-associated isolates (Eaton and Gasson, 2001; Franz *et al.*, 2001; Semedo *et al.*, 2003).

Enterococci harboring some of these virulence factors have been described so far in traditional Portuguese dry fermented sausages (Ribeiro *et al.*, 2011), ewe's raw milk cheeses (Mannu *et al.*, 2003), traditional Italian cheeses (Maietti *et al.*, 2007), ewes' cheese and milk from four Portuguese Registered Designation of Origin areas (Semedo *et al.*, 2003), among others.

1.4.2. Susceptibility to antibiotics

One of the many causes for enterococci being of major concern in human health is the fact that they are resistant to different antibiotics. This poses as a selective advantage, especially in environments such as hospitals, were antimicrobial agents are heavily used, eliminating susceptible and competing bacteria. Antibiotic usage allows enterococci to spread and cause healthcare-associated infections. In general,

enterococcal antibiotic resistance has been most frequently investigated in human clinical isolates, due to the direct impact (Ogier and Serror, 2008).

Antimicrobial resistance can be divided into inherent or intrinsic and acquired. The terms inherent or intrinsic resistance indicate a usual resistance present in all or most of the strains of a given species. The genes for intrinsic resistance usually reside on the chromosome. The various intrinsic (inherent) antibiotic resistance traits in enterococci include resistance to semisynthetic penicillinase-resistant penicillins, cephalosporins, low levels of aminoglycosides, and low levels of clindamycin.

Acquired resistance is a consequence from either a mutation in the existing DNA or acquisition of new DNA. In general, acquisition of DNA occurs by transformation (a process not known to occur in nature with enterococci), transduction (a process recently suggested for enterococci), or conjugation (Murray, 1990).

Examples of enterococcal acquired resistances include resistance to chloramphenicol, erythromycin, high levels of clindamycin, tetracycline, high levels of aminoglycosides, penicillin (by means of penicillinase), fluoroquinolones and vancomycin.

Aminoglycosides, such as gentamicin and streptomycin, target protein synthesis, by linkage to the 30S rRNA subunit (Werner, 2011). Low-level aminoglycoside resistance among *E. faecalis* strains can be associated with the *aac6'-aph2"* (*aac(6')-Ie-aph(2")-Ia*) gene that encodes a bifunctional enzyme encoding high-level resistance to all aminoglycosides except streptomycin (Horodniceanu *et al.*, 1979). It is the most prevalent form of acquired gentamicin resistance in both *E. faecalis* and *E. faecium*. Low level of gentamicin resistance can also be associated with decreased permeability to the antibiotic (Werner, 2011). High level gentamicin resistance may be encoded by determinants such as *aac(6')-Ii, aph(2")-Ie*, and *ant(6)-Ia*, that codify to aminoglycoside-modifying enzymes (Jackson *et al.*, 2004; Mahbub *et al.*, 2005).

β-Lactams, resemble the dipeptide D-alanine, D-alanine and can connect to the penicillin binding proteins (PBPs). They target the cell wall synthesis by inhibiting the transpeptidase (Werner, 2011).

The resistance of enterococci to β-lactams is a characteristic feature that appears to be related to the low affinity of their penicillin-binding proteins (*pbp's*). The most common *pbp* in enterococci is the Pbp5. In the specific case of Pbp5, the affinity reduction is related with point mutations, the addition of aspartic acid or serine after position 466 and change of methionine to alanine or threonine at position 485, alanine or isoleucine to threonine at position 499 and glutamate to valine at position 629 were found to be significantly associated with ampicillin resistance (Hsieh *et al.*, 2006). Although rare, the resistance to β-lactams in *E. faecalis* may also be associated with β-lactamases (Werner, 2011).

Phenicols inhibit protein synthesis by binding to the 50S rRNA subunit. The main resistance mechanism in enterococci involves the production of an enzyme, chloramphenicol-acetyltransferase, which modifies the antibiotic by inactivating it (Werner, 2011).

Glycopeptides, like vancomycin and teicoplanin, block the incorporation into the peptidoglycan of subunits of N-acetylmuramic acid and N-acetylglucosamine, by binding reversibly to these molecules. In enterococci the main resistance mechanism involves the production of 'abnormal' peptidoglycan precursors (resulting in production of D-Ala-D-lactate/D-Ala-D-serine instead of D-Ala-D-Ala), for which vancomycin has reduced affinity (Werner, 2011).

Resistance to vancomycin and teicoplanin can be mediated by plasmids (*e.g.* pIP819 and pIP821) (Leclercq *et al.*, 1989). Two different transferable vancomycin-resistant phenotypes are known, the VanA

and the VanB (Giraffa, 2002). The vancomycin resistance gene *vanA* is predominantly present in Tn3 transposons derivate *Tn1546* (Wegener *et al.*, 1999; Coque *et al.*, 2011).

Vancomycin-resistant enterococci (VRE) in particular, pose a major problem in treating human infections since vancomycin is used as last choice antibiotic for severe infections where the host has a compromise immune system and penicillins are not being effective (Klein, 2003).

The first VRE was isolated in Europe, in France, in 1986; and in 1989 in the USA, where rapidly became frequently associated with healthcare-associated infections. In the USA, VRE infections are probably associated with the heavy use of antibiotics in hospital, which is higher than in Europe.

At first, regarding with the USA outbreak, it was thought the VRE phenotype was also associated with a hospital environment. However, in Europe the healthcare-associated infections did not increase at the same rate. Besides, in 1998 there were reports in the United Kingdom of VRE from pigs herds. These facts lead to the suggestion that the VRE had a source in the community and could be associated with the use of glycopeptides as growth promoters in food animals (Wegener *et al.*, 1999). Overall, it appears that a large reservoir of transferable antibiotic resistance in various ecosystems was created and could be related to the emergence of glycopeptides resistance in *E. faecium* outside hospitals (Giraffa, 2002).

Avoparcin was first approved as a growth promoter in Europe in 1974 and the first VRE was detected in 1986, although if no VRE was found before it could have been because no one looked for it. Until 1997 in the European Union, the glycopeptide, avoparcin was used as a growth promoter in animal feed. Wegener *et al.* (1999) suggested that the use of avoparcin as a growth promoter is associated with the occurrence of VRE in food animals, especially in Europe.

Glycopeptide resistant enterococci (GRE) could therefore have been introduced in the food chain and might be transferred via food to the human gut. An additional risk could be the transfer of these resistances from GRE to other gut bacteria by conjugation (Klein, 2003). The transfer of genetic resistance to vancomycin to other Gram positive bacteria has already been demonstrated *in vitro* by Noble *et al.* (1992).

Macrolides, such as erythromycin, bind to the 23S rRNA inhibiting elongation of the protein by peptidyl transferase, preventing translocation of the ribosome or both. In enterococci the main resistance mechanism involves the production of enzymes that modify the target, prevent or limit binding of the agent (Werner, 2011).

Different erythromycin resistance determinants have been described, but the most common is *erm(B*), carried by a transposon widespread in human and animal isolates. Erythromycin resistance can occasionally be encoded by the *erm(A)* or *erm(C)* genes. The erythromycin resistance methylases confer resistance by modifying the 23S rRNA (Roberts *et al.*, 1999). The *erm(B)* determinant is widespread among enterococci, especially *E. faecium* and *E. faecalis*, and is part of many multi-resistance plasmids and often linked to Tn*1546*-like *vanA* elements (Borgen *et al.*, 2002). Tn917 that contains the *erm(B)* gene is often located on the hemolysin/bacteriocin plasmid of *E. faecalis* pAD1 (Coque *et al.*, 2011).

Quinolones such as nalidixic acid, inhibit the topoisomerase II (gyrase) and topoisomerase IV, essential for cell replication (Jacoby, 2005). The mechanisms involved in quinolone resistance include mutations that alter the drug's target; reduce drug accumulation or plasmids that protect the bacterial cell from quinolones. The quinolone targets are the bacterial enzymes DNA gyrase and DNA topoisomerase IV (Werner, 2011).

Sulphamethoxazol and trimethropim inhibit the folic acid production. In enterococci the main resistance mechanisms involve target production (enzyme in the metabolism of folic acid) in excess; and reduced accumulation of the agent due to the operation of efflux pumps (Werner, 2011).

Tetracyclines block the binding of tRNA to the 30S rRNA subunit. In enterococci the main resistance mechanisms are the so called 'ribosomal protection genes' encoding for proteins that protect the ribosome by preventing binding of the agent; and reduced accumulation of the agent due to the operation of efflux pumps (Werner, 2011). Tetracycline resistance in most bacteria is due to the acquisition of new genes, often associated with mobile elements. These genes are usually associated with plasmids and/or transposons and are often conjugative (Roberts *et al.*, 2005).

There are several acquired tetracycline resistance genes already described. From these, some encode energy-dependent efflux proteins, *tet(A)*, *tet(B)*, *tet(C)*, *tet(D)*, *tet(G)*, *tet(H)*, *tet(K)* and *tet(L)*, while others encode for ribosomal protection proteins, *tet(M)*, *tet(O)*, *tet(S)*, *tet(Q)* and tet(W), or even tetracycline inactivating enzymes. The most common gene identified in 42 different genera (*Enterobacter*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Lactococcus*, *Microbacterium*, Mycobacterium, *Neisseria*, *Pseudomonas* and *Streptomyces*) is *tet(M)*. This gene is frequently associated with conjugative transposons, namely Tn916 and Tn1545 (Roberts, 2005).

Barbosa *et al.* (2009) described the antibiotic resistance profile of enterococci isolated from Portuguese traditional fermented meat products. These authors found that the meat-enterococci were susceptible to ampicillin and penicillin, ciprofloxacin and chloramphenicol. Some of the enterococci were resistant to erythromycin, nitrofurantoin, rifampicin and tetracycline. None of the enterococci were vancomycin resistant. A more complete summary regarding the antibiotic resistance of enterococci isolated from these and other food products can be seen in Table 3.

Table 3 - Reported incidences of antibiotic resistances among *E. faecium* and *E. faecalis* strains isolated from foods (Franz *et al.*, 2011).

Resistance to antibiotic	% Incidence of resistance of enterococci from foods									
	Fermented sausages[1]	fermented sorghum[2]	cheese/ other foods[3]	retail meats (turkey / chicken)[4]	retail meats (pork/beef)[4]	produce[5]	ready to eat foods[6]	Bryndza cheese[7]	Moroccan foods[8]	Pecorino Abruzzese cheese[8]
for *E. faecium* strains:	(n=55)	(n=22)	(n=48)	(n=213/245)	(n=114/245)	(n=97)	(n=47)	(n=178)	(n=15)	(n=34)
ampicillin	30.9	0	0	54/23	4.4/2.8	nd	6.4	0	0	3
penicillin	58.2	9.1	45.8	53/20	9.6/8.7	7	12.8	nd	0	36.4
erythromycin	56.4	31.8	27.1	87/43	60/39	10	29.8	36	67	75.7
tetracycline	29.1	0	6.3	0.9/0.4	0.9/0.4	29	17	nd	7	3.0
chloramphenicol	20	0	10.4	41/22	7/19	5	2.1	nd	0	39.4
ciprofloxacin	54.5	13.6	56.3	nd	nd	28	25.5	2	67	27.3
gentamicin	0	0	2.1	nd	nd	0	nd	0	0	12.2
streptomycin	nd	0	4.2	nd	nd	3	nd	0		3
vancomycin	1.8	13.6	2.1	nd	nd	0	0	0	7	0
rifampin	69.1	nd	nd	nd	nd	nd	19.1	31	27	nd
for *E. faecalis* strains:	(n=5)	na	(n=47)	(n=110/51)	(n=161/66)	(n=38)	(n=52)	(n=49)	(n=23)	(n=28)
ampicillin		na	2.1	nd	nd	nd	0	0	0	0
penicillin		na	12.8	0	0/0	0.6/0	0	nd	0	37.9
erythromycin	93.3	na	63.8	3	42/33	8.1/4.5	26.4	22	22	48.3
tetracycline	86.7	na	44.7	0	94/67	89/39	41.5	nd	87	
chloramphenicol	93.3	na	31.9	3	0/0	3.1/0	24.5	nd	9	6.9
ciprofloxacin	46.7	na	27.7	5	0/0	0.6/0	0	2	61	0
gentamicin	0	na	25.5	0	nd	nd	nd	0	0	6.9
streptomycin	nd	na	46.8	0	nd	nd	nd	0	17	0
vancomycin	0	na	0	0	nd	nd	0	0	9	0
rifampin	100	na	nd	nd	nd	nd	11.3	29	78	nd

1.5. Enterococcal Biofilms

Biofilms are a protective mode of growth that allows bacteria to survive in hostile environments, such as manufacturing and hospital settings.

A biofilm is a population of cells attached irreversibly to a biotic or abiotic surface, encased in a hydrated matrix of exopolymeric substances, proteins, polysaccharides and nucleic acids. Inside the biofilm structure, nutrients flow and different cells are organized spatially exhibiting different patterns of gene expression. Biofilms can give rise to nonsessile individuals, planktonic bacteria that can rapidly multiply and disperse. These resistance structures develop preferentially on inert surfaces, or on dead tissue, occurring commonly on medical devices.

The pattern of development (Fig. 4) involves initial attachment to a solid surface, the formation of micro-colonies (relatively small groups of bacteria) on the surface, a maturation step of differentiation of micro-colonies into exopolysaccharide-encased and into a mature biofilms, and finally the detachment and dispersal of planktonic cells to allow the colonization of new areas (Costerton *et al.*, 1999).

The regulation of gene expression in biofilms is accomplished by quorum sensing, a cell population density response mechanism (Mohamed and Huang, 2007).

Numbers concerning the prevalence of enterococcal biofilms differ geographically. Mohamed and Huang (2007) summarized various reports on the prevalence of enterococcal biofilms and the numbers vary, but one thing is maintained, the fact that the two major species involved are *E. faecalis* and *E. faecium*.

Biofilms can also form on living tissues, as in the case of endocarditis. Biofilms grow slowly, in one or more locations, and biofilm-associated infections are often slow to produce evident symptoms. Sessile bacterial cells release antigens and stimulate the production of antibodies, but the antibodies are not effective in killing bacteria within biofilms and may cause immune complex damage to surrounding tissues.

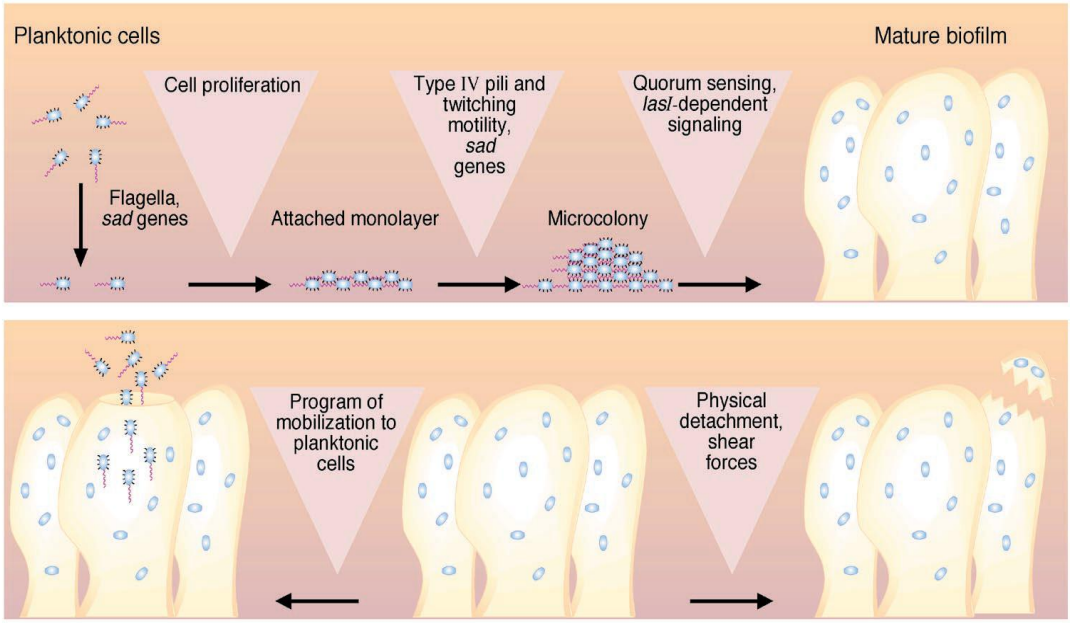

Figure 4 - Model of the development development of a mature *Pseudomonas aeruginosa* biofilm from planktonic cells; dispersal of bacteria from a biofilm (Costerton *et al.*, 1999).

Biofilms are difficult to eradicate and can be a source of many chronic infections (Costerton *et al.*, 1999; Mohamed and Huang, 2007), such as bacterial endocarditis, infectious kidney stones and cystic fibrosis, but they are also central to nosocomial acquired infections related to medical devices (Tendolkar *et al.*, 2004).

Several factors can influence biofilm production. Nutrients in the growth medium, like glucose, serum, iron and CO_2 play a major role in the production of the biofilm's structure. Temperature, osmolarity and pH can also be of great influence (Mohamed and Huang, 2007).

To date a group of genes have been reported to be involved in the early steps of attachment and biofilm formation in enterococci, namely *esp*, *ebpABC*, *fsr*, *gelE* and *epa*.

Since biofilms are complex communities with individual bacterial cells embedded in an extracellular polysaccharide matrix they are inherently resistant to antimicrobial treatment that mostly cannot penetrate this matrix, turning these structures a major concern in the hospital settings.

Biofilm formation is also a matter of concern in food industries, because it facilitates the survival of pathogens that can contaminate food processing equipments and products (Paganelli and Leavis, 2011). Biofilm producing enterococci have been described in small quantities between other LAB inside wooden vats, used to make Ragusano Sicilain cheese; and wooden shelves, used to store ripening French cheeses (Paganelli and Leavis, 2011).

Under certain environmental conditions enterococcal cells may switch from planktonic to biofilm mode. Since enterococci are known for their mutiresistance to antibiotics, biofilm formation associated with infections further hampers treatment. Although enterococcal isolates from food have been demonstrated to be biofilm producers, there is no knowledge whether ingestion of enterococcal biofilm could lead to health problems.

1.6. Aims of the study

Enterococci are ubiquitous bacteria, part of the autochthonous gastrointestinal microbiota of humans and other animals; can also be found in soil, plants and water. More importantly, for this study, they are frequently found in foods, especially in fermented products.

Even though they are commensal bacteria, over the last decades enterococci have been described as opportunistic pathogens associated with several life-threatening infections. This pathogenicity is associated with the presence of several virulence factors (*agg*, *efaAfs*, *esp*, *gelE*, *cylA*, *epa*, *fsrABC*) as well as their (intrinsic or acquired) antibiotic resistance. The pathogenicity potential has not only been described for clinical enterococcal isolates but there have also been reports regarding food-enterococci.

In the Mediterranean countries, such as Portugal, fermented meat products are a very common "delicacy", produced in an artisanal way. Since enterococci are frequently associated with fermented (meat) products in the present study first we aimed to investigate (1) the presence of enterococci in traditional Portuguese fermented meat products; and (2) the main species present. In order to adress these questions we investigated the presence of enterococci in traditional fermented meat products, such as Catalão, Chouriço Preto, Linguiça, Salsichão and Paio.

Since most of the organoleptic traits of traditional fermented meat product come from LAB fermentation, and several enzymatic activities are attributed to enterococci, we wanted to understand if (3)

meat-enterococci have technological potential? and (4) which enzymatic activities do meat-enterococci possess?

Also attributed to enterococci is the ability to produce bacteriocins, so we wanted to (5) understand if meat-enterococci could be bacteriocin producers and (6) which bacteriocin(s) do they produce? and (7) what is the main antimicrobial spectrum of action of said bacteriocin(s)?

To answer these questions, we screened the meat-enterococci for enzymatic activities and bacteriocin production against five pathogens that included *L. monocytogenes*, *E. coli*, *Salmonella enterica* serovar Enteritidis and vancomycin resistant *E. faecium* and *E. faecalis*. Afterwards, we screened, through PCR-amplification, for the presence of determinants encoding five common enterocins (enterocin A, enterocin B, enterocin L50 A/B, enterocin P).

Since these products are largely consumed and enterococci have been described as human pathogens this raised some additional questions; (7) do meat-enterococci carry virulence traits? (8) are they resistant to antibiotics? (9) can they produce biofilms that may allow them to persist in the manufacturing environment?

The present study aimed to answer these questions. In order to do so, we selected for analysis some of the most commonly described virulence factors for enterococci; and fourteen different antibiotics, representing eight different classes, for the analysis of antibiotic resistance. Furthermore, we analyzed the ability of the meat-enterococci to produce biofilms in a polystyrene surface.

The answers to these questions should help to understand the duality of meat-enterococci, associated with the fact that even though they typically harbor virulence factors and antibiotic resistances, there has never been a report of an enterococcal infection directly associated with the consumption of food product harboring these bacteria. Moreover, this study can help understand the extent to which the use of enterococci in food technology is safe for human health.

2. Materials and Methods

2.1. Samples and enterococcal isolation

The samples under analysis consisted of various types of traditional fermented meat products, namely Catalão, Chouriço Preto, Linguiça, Salsichão and Paio, obtained from a processing unit located in Alentejo. Once in the laboratory all samples were processed according to International Standards Organization Method 7899-2 (Anonymous, 2000) with minor alterations.

For each sample 25 g were mixed with 225 ml of buffered peptone water solution (Scharlau, Barcelone, Spain). The mixture was homogenized in a Stomacher blender (Stomacher Lab-Blender 400) and decimal dilutions were spread on Slanetz and Bartley Agar (SBA) (Scharlau, Barcelone, Spain) and incubated at 42ºC for 48 hours. For these isolation procedures we used both SBA and SAB supplemented with 10 μg/ml of vancomycin. After incubation, at least 5 typical colonies from each sample/medium were collected, their ability to hydrolyze esculin was assessed by growth in Bile Esculin Azide Agar -BEAA- (Scharlau, Barcelone, Spain) and they further purified by streaking in SBA. After a minimum 5 step purification, the isolates were stored in Brain Heart Infusion -BHI- broth (Scharlau, Barcelone, Spain) containing 20% (v/v) glycerol at -80ºC. For routine use, enterococcal isolates were cultivated on BHI broth or plates, at 37ºC.

2.2. Genus/Species Identification and Genomic Typing

Presumptive identification of the isolates at genus level was carried out through the next initial steps: observation of colony characteristics both in SBA and BEAA, observation of cell morphology; Gram staining; catalase and oxidase production; growth at 10ºC, 37º C and 45º C; growth in the presence of 6.5% NaCl and at pH 9.6.

To evaluate growth ability the isolates were incubated in a 96 well microtitre plate under the referred conditions. The isolates were inoculated in 200 µl of Tryptic Soy Broth (TSB) (Scharlau, Barcelone, Spain). After an incubation period of 24 hours, increase in turbidity was indicative of growth (Devriese *et al.*,1993). For each plate, non-inoculated medium was used as a sterility control.

To further confirm that all isolated belonged to the *Enterococcus* genus, we preformed a PCR-amplification according to the method described by Ke *et al.* (1999) (Appendix A).

Identification at species level was performed by multiplex-PCR using species specific primers (see Table 4 and Appendix A). Each PCR reaction was carried out using a mixture containing 1X reaction buffer (NZYTech, Lda, Lisboa, Portugal), 0.2 mM of each dNTP (NZYTech, Lda, Lisboa, Portugal), 3 mM $MgCl_2$ (NZYTech, Lda, Lisboa, Portugal), 2 *µ*M of each primer (NZYTech, Lda, Lisboa, Portugal), 1 U of Taq polymerase (NZYTech, Lda, Lisboa, Portugal) and approximately 100 ng of DNA. The primers were combined as shown in Table 4. Amplification conditions are described in Appendix A.

Table 4 - Multiplex-PCR combinations used for identification at species level.

Combination	Primers	Target Species
1	ddlE1 + ddlE2 FM1 + FM2	*E. faecalis*
2	DU1 + DU 2 ddlF1 + ddlF2	*E. durans* *E. faecium*
3	mur2F + mur2 R FL1 + FL2	*E. hirae* *E. faecium*
4	CA1 + CA2	*E. casseliflavus*

Genomic typing involved a PCR-fingerprinting using the primers $(GTG)_5$ and OPC19 in independent reactions, according to Ŝvec *et al.* (2005) and Ribeiro *et al.* (2011). For the amplification 1 µl of DNA (approximately 100 ng) was transferred to 25 µl of PCR mixture containing 1X reaction buffer , 3 mM MgCl2, 0.2 mM of each dNTP , 2 *µ*M of primer, either primer $(GTG)_5$ or OPC19 (Appendix A), 1 U of Taq polymerase. PCR amplifications were performed under the conditions described in Appendix A.

The PCR reactions were performed in a Thermocycler (Doppio, VWR, Radnor, Pennsylvania, USA). For all the PCR products, 8 *µ*l along with 2 *µ*l of loading buffer, were resolved by agarose gel electrophoresis (1.2% [w/v] in 0.5X TBE at 90 V for 2h30m. On each gel, a molecular weight marker (1 Kb Plus, Invirtogen, Life Technologies) was included at two positions. All gels were photographed on ImageMaster (PharmaciaBiotech, GE Healthcare, UK).

BioNumerics software (version 6.6, Applied Maths, Kortrijk, Belgium) was used to register PCR fingerprinting patterns, normalize densitometric traces, calculate the Pearson product–moment correlation coefficient, and perform cluster analysis by the unweighted pair group method with arithmetic mean algorithm (UPGMA). The reproducibility level was assessed by analyzing a random sample of 10% duplicates.

The intra-specific diversity was also calculated using the Simpson's (D) (Hunter *et al.*, 1988).

$$D = \sum_{i=1}^{S} \frac{n1\ (n1-1)}{N\ (N-1)}$$

S- total number of groups formed; N – total number of isolates analyzed; n – number of isolates in the group.

2.3. Food technological potential

2.3.1. Bacteriocinogenic properties

Potential bactericin-producer strains were grown overnight at 37ºC in BHI and after centrifugation at maximum velocity (Eppendorf Centrifuge 5415R) for 30 min, the cell-free supernatant was recovered to a new tube. Production of antimicrobial substances was tested by spotting 7 µl of the cell-free supernatant over a BHI plate, where the indicator strain was previously swabbed, followed by incubation at 37ºC for 24 h.

Indicator bacteria included the vancomycin-resistant *E. faecium* E300 and *E. faecalis* V583, *Escherichia coli* DSMZ 8739, *Listeria monocytogenes* CECT 935, *L. monocytogenes* 910, and *Salmonella enteric* serovar Enteritidis LX 3-11.

Structural genes (*entA, entB, entL50A/B and entP*) coding for different bacteriocins (enterocin A, B, L50 A/B and P, respectively) were screened by PCR in all presumptive bacteriocin producer enterococci, using primers and conditions previously described by Pangallo *et al.* (2004) (Appendix A). For each reaction the next positive controls were used: AS34b (positive control for L50AB and enterocin P) and AV25a (positive control for enterocin A and B).

2.3.2. Enzymatic activities

Lipase activity was detected with Spirit Blue Agar and Lipase Reagent (Difco, Franklin Lakes, USA). Colonies of lipolytic organisms were recognized by a dark-blue color to a clearing beneath and surrounding the colonies (a kind of a halo effect).

Hydrolysis of casein was determined by spot inoculation of strains on agar plates containing skimmed milk powder (20 g/l) and agar (15 g/l). Caseinolytic activity was detected as clear zones around the spots after incubation of plates at 37ºC for 48 h.

To access the ability to produce other enzymes with food technological potential the Apy-Zim kit (BioMérieux, Marcy-L'Etoile, France) was used, following the manufactures instructions. This kit allows screening the production of 19 different enzymes (alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, napthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucoronidase, α- gluconidase, β-gluconidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase).

2.4. Pathogenicity potential

2.3.3. Virulence factors

The presence of some of the most frequent and best described enterococcal virulence factors was screened by PCR amplification and/or plate assays. This included the coding genes for aggregation substance (*agg*), the *E. faecalis* antigen A (*efaAfs*), the enterococcal surface protein (*esp*), the pili-like

(*ebpABC*); gelatinase (*gelE*); gelatinase regulator (*frsB*); cytolysin (*cylA*) and the enterococcal polysaccharide antigen gene (*epa*). The primers used are described in Table 5 and Appendix A. *E. faecalis* MMH 594 was used as control strain.

Table 5 - Multiplex-PCR combinations used to screen for virulence determinants.

Combination	Primers
1	*cylA* F + R *esp* F + R *agg F* + R *gelE* F + R
2	*ebpA* F + R *ebpB* F + R *ebpC* F + R *efaAfs* F + R
3	*epa* F + R *fsrB* F + R

Each PCR reaction was carried out using a mixture containing 1X reaction buffer, 0.2 mM of each dNTP, 3 mM $MgCl_2$, 2 *μ*M of each primer , 1 U of Taq polymerase and approximately 100 ng of the isolates DNA. The amplification conditions are described in Appendix A. The amplification was carried out in Thermocycler (Doppio, VWR, Radnor, Pennsylvania, USA). The PCR products (8 *μ*l) along with 2 *μ*l of loading buffer were resolved by agarose gel electrophoresis (1.2% [w/v] in 0.5X TBE at 90 V for 2h 30m. After staining in a GelRed bath (Biotium, Inc, Hayward, Canada), gel images were acquired with ImageMaster (PharmaciaBiotech, GE Healthcare, UK).

Regarding the plate assays: Hemolysin activity was determined on Columbia Blood Agar (Oxoid, Basingstoke, England) containing 5% defibrinised horse blood after 48 h of incubation at 37 ºC on anaerobiosis. Zones of clearing around colonies indicate β-hemolysin production.

Gelatinase activity was detected with a 3% gelatin medium: meat peptone (5 g/l), yeast extract (3 g/l), gelatin (30 g/l), agar (15 g/l), pH 7. After incubation at 37 ºC for 48 h, the plates were overlaid with a solution of ammonium sulphate (300 g/l in distilled water). The producers of gelatinase were detected by the formation of a transparent halo.

2.3.4. Susceptibility to antibiotics

Susceptibility to fourteen antimicrobial agents (see Table 6) was evaluated by the disk diffusion method, using breakpoints of resistance previously established by the National Committee for Clinical Laboratory Standards (2008). Breakpoints applied are gathered on Appendix B.

MICs for vancomycin were determined using the Etest (Oxoid Limited, Cambrige, United Kingdom) strips, following the manufactures' instructions.

Table 6 - Antibiotics used in this study.

Class		Antibiotic	Symbol	Disc content (µg)
Aminoglycosides		Gentamicin	CN	10 and 120
		Streptomycin	S	10
β - Lactams	Penicillins	Ampicilin	AMP	10
		Amoxycillin/Clavulanate	AMC	30
		Penicillin G	P	10 units
	Cephalosporins	Cefalexin	CL	30
		Cephotaxim	CTX	30
Phenicols		Chloramphenicol	C	30
Glycopeptides		Vancomycin	VAN	30
Macrolides		Erythromycin	E	15
Quinolones		Nalidixic Acid	NA	30
Sulphonamides/Pyrimidins		Sulphamethoxazole/Trimethropim	STX	25
Tetracyclines		Tetracycline	TET	30

Some of the most common enterococcal genes associated with antibiotic resistance were screened by PCR (*erm(B)* for erythromycin resistance, *tet(M)* for tetracycline and *vanA* and *vanB* for vancomycin resistance). For each reaction a positive control was used: *E. faecalis* V583 (positive control for *vanB* and *ere(B)* and *E. faecalis* AR01/DG (positive control for *vanA*, *ere(B)* and *tet(M)*), see Appendix A for details. Each PCR reaction was carried out using a mixture containing 1X reaction buffer, 0.2 mM of each dNTP, 3 mM $MgCl_2$, 2 *µ*M of each primer, 1 U of Taq polymerase and approximately 100 ng of the isolates DNA. The PCR products (8 *µ*l) along with 2 *µ*l of loading buffer were resolved by agarose gel electrophoresis (1.2% [w/v] in 0.5X TBE at 90 V for 2h 30m. After staining in a GelRed bath, gel images were acquired with ImageMaster (PharmaciaBiotech, GE Healthcare, UK).

2.3.5. Enterococcal Biofilms

For the detection of biofilm-producing enterococci the Extremina *et al.* (2010) microtitre-plate of 96 wells protocol was used, with minor alterations. An overnight culture of the enterococcal isolates was grown in Tryptic Soy Broth (TSB) at 37ºC. The optical density (OD) was measured to ensure a cellular concentration of 10^9 CFU/ml. The bacteria in the corresponding volume were then washed by centrifugation for 15 min at maximum speed. The supernatant was discarded, the pellet resupended in 1 ml of PBS (8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4 and 0.24 g/l KH_2PO_4) and centrifuged for 5 min at maximum speed. This step was repeated two more times. Lastly, the bacterial cells were resuspended in a volume of 100 µl of PBS. Meanwhile, 200 µl of TSB + 0.5% glucose (Scharlau, Barcelone, Spain) were added to each well in the microtitre-plate. Each well was inoculated with 2 µl of the bacterial suspension previously prepared to a final concentration of 2% and plates were incubated at 37 ºC for 48 h. Three repetitions were made for each bacterium. Some wells weren't inoculated (blanks) to allow a sterility control of the assay. The positive control consisted in the inoculation of *E. faecalis* MMH 594, a known biofilm producer.

After the incubation period the biomass quantification was performed by the crystal violet staining method. In this method all the medium was completely removed from the wells, followed by a series of three washing steps with PBS and drying at room temperature for 1h (inside a flow chamber). Then, a drop of a solution of 0.1% of crystal violet was added for 15 min (room temperature). The stain was completely removed and the wells were subsequently rinsed three times with PBS to remove remaining dye. The plates dried for 15 min at room temperature and the dye bound to the biofilms was extracted

using acetone/ethanol (80:20). After 15 min the Abs_{590nm} was measured using a micro-ELISA plate reader (Thermo Fisher Scientific Inc, Langenselbold, Germany).

In Fluorescent *in situ* hybridization (FISH) protocol the EUB 338 probe (5'-TGAGGATGCCCTCCGTCG-3') (Amann *et al.*, 1990), which is complementary to a portion of the 16S rRNA gene conserved in the domain *Bacteria*, was used to visualize the entire bacterial population.

An overnight culture of the enterococcal strains was grown in BHI broth at 37 ºC. The optical density (DO) was measured to ensure a cellular concentration of 10^8 CFU/ml. The bacteria in the corresponding volume were then washed, by centrifugation for 15 min at maximum speed. The supernatant was discarded and the pellet resupended in 1 ml of PBS and centrifuged for 5 min at maximum speed. This step was repeated two more time. Lastly the bacterial cells were resuspended in a volume of 100 µl of BHI broth. The cultures were diluted 1:10 and 1:100. From this bacterial suspension, 10 µl were smeared on a glass slide and incubated for 24h and 48 h at 37 ºC.

After the incubation time, the cells on the glass slides were fixed with the addition of 10 µl per spot of a 4% paraformaldehyde in PBS (v/v), for 2h at room temperature. The glass slides were them washed with water and dried at room temperature. The next step was a series of dehydrations with 50%, 80% and 96% ethanol solutions for three minutes each. To allow permeabilization of the cell membrane, the glass slides were treated with 10 µl of lysozyme (0.01, 0.1 and 1 mg/ml) prior to hybridization, for 4, and 10 minutes at room temperature. This step was followed by seriated dehydration with ethanol. For the hybridization, 10 µl of the hybridization buffer (0,9 M NaCl, 20 mM Tris-HCl (pH 7,2), 0,01% SDS and EUB 338 5 ng/*µ*l) were added to each spot, and incubated at 45ºC for 3h. The smears were incubated under a cover-slip in a buffer-saturated chamber (Omnislide Thermal Cycler In Situ PCR Machine, Humidity chamber, ThermoHybaid). Finally, the glass slides were washed with 10 µl of washing solution (0,9 M NaCl, 20 mM Tris-HCl (pH 7,2) and 0,1% SDS) for 15 min at 45 ºC.

After being air dried and mounted in Vectashield (Vector Laboratories, H1200) containing DAPI (4'-6-diamino-2-fenilindol). The slides were analyzed on a with an DMR (Leica) epifluorescence microscope.

3. Results and Discussion

3.1. Species identification and strain diversity of the meat-enterococci

Enterococci are a ubiquitous group of bacteria, which are a part of the autochthonous microbiota present in the gastrointestinal tract of humans and other animals. Enterococci are also frequently found in food, especially fermented products. Even though they are commensal bacteria, over the last decades enterococci have been described as opportunistic pathogens associated with several life-threatening infections. Since enterococci are frequently associated with fermented meat products these combination of factors can raise some concern regarding human health.

The present investigation studied several Portuguese traditional fermented meat products namely, Catalão, Chouriço Preto, Linguiça, Paio and Salsichão, for the presence of *Enterococcus* spp.

The initial step of isolation was performed in SBA. This medium is used for the detection and enumeration of enterococci in water (ISO 7899-2). Since some authors (Cetinkaya *et al.*, 2000) suggest that some

VRE are vancomycin dependent, we used a variation of the isolation media without vancomycin and another supplemented with 10 µg/ml of vancomycin. A presumptive positive result is the growth red to pink colonies, since enterococci reduce TTC to formazan inside the cells. We collected, from the lowest countable dilution, five colonies corresponding to the characteristic growth and whenever possible, representing different colony morphologies.

Next, the isolates were grown in BEAA (ISO 7899-2), to confirm characteristic growth. Organisms positive for esculin hydrolysis, like enterococci, hydrolyze the esculin to esculetin and dextrose. The esculetin reacts with the ferric citrate to form a dark brown or black complex, visible around the bacterial growth in the medium. Only the isolates with characteristic growth in both media were selected and purified.

To further confirm genus identification the isolates were tested for the criteria which are generally considered to define enterococci: the ovoid cell morphology, Gram-positive, catalase and oxidase negative, and growth at 10ºC, 37º C and 45º C, at presence of 6.5% NaCl and at pH 9.6. Only the ones following these criteria were presumably classified as *Enterococcus* spp.

Since the phenotypic methods have proven not to be enough for secure identification, a molecular approach was taken. In the molecular confirmation of the genus a PCR-amplification was carried out according to the method described by Ke *et al.* (1999). According to this method the amplification for enterococci should result in a 112 bp product (Figure 6). A total of 148 isolates were positive for these amplification product and were classified as *Enterococcus* spp.

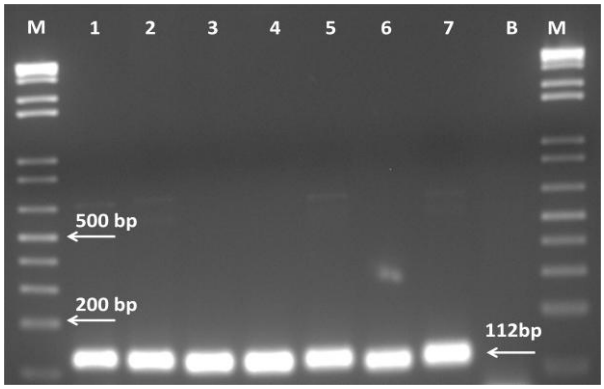

Figure 5 - Example of the genus PCR-amplification. Lanes: 1, *E. faecium* DSMZ 2146; 2, *E. faecium* DSMZ 20477; 3, *E. faecalis* DSMZ 20478; 4, *E. faecalis* DSMZ 20376; 5, *E. hirae* DSMZ 20160; 6, *E. casseliflavus* DSMZ 20680; 7, *E. durans* DSMZ 20633; B, Blank; M. 1 kb Plus molecular size standard ladder.

Overall, the frequency of enterococci was approximately 10^4 CFU/g for Catalão, Chouriço Preto and Linguiça, both for SBA without vancomycin and SBA supplemented with vancomycin. For Salsichão and Paio, the frequency of enterococci was below 10^2 CFU/g.

In Portugal, fermented sausages can have high counts of *Enterococcus* spp., ranging from 10^4 to 10^8 CFU/g, while others possess lower numbers of approximately 10^3 to 10^4 CFU/g (Ribeiro *et al.*, 2011). These reports are according to our findings.

Since, several colonies from the same products and plate were collected, it was essential to guarantee that we were not working with clones. To do so, we resorted to a genomic typing technique. The genetic diversity of the 148 meat-enterococci was assessed by PCR-fingerprinting with primers OPC-19 and $(GTG)_5$. And a dendrogram was constructed from the patterns obtained (data not shown). The

reproducibility level of 10% replicates was found to be 90%, and a cutoff value of 80% similarity was chosen, based on the visual analysis of the clusters to calculate Simpson's diversity index and selective representative strains. This led to the selection for further studies of 75 meat-enterococci, representative of all enterococcal groups and products.

The Simpson's diversity was D=0.927. Simpson's index is interpreted as the possibilities of two randomly selected organisms belong to the same type. The maximum value possible (1) corresponds to one individual per group. Since the diversity value obtained is close to 1, we can assume our sampling allowed to capture the diversity of the products under study.

Subsequently, for the identification at species level a multiplex-PCR was performed. Table 7 summarizes the expected amplification products sizes for the tested species. Figure 6 and 7 exemplify the results observed.

Table 7 - Expected amplification products sizes.

Species	Primers	Amplicon size (bp)
E. faecalis	ddlE1 + ddlE2 FM1 + FM2	941 215
E. faecium	ddlF1 + ddlF2 FL1 + FL2	550 360
E. durans	DU1 + DU 2	295
E. hirae	mur2F + mur2 R	521
E. casseliflavus	CA1 + CA2	288

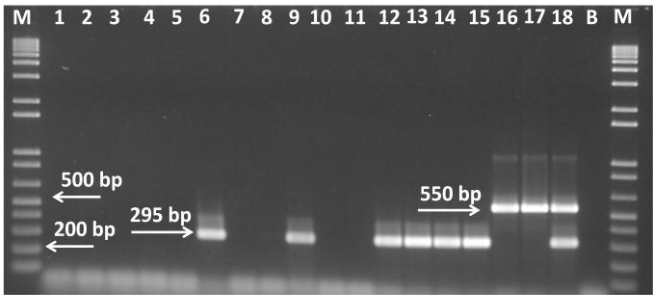

Figure 6 - Example of the species PCR-amplification. Lanes: 1 to 14, meat-enterococci; 14, *E. durans* DSMZ 20633; 15, *E. faecium* DSMZ 2146; 16, *E. faecium* DSMZ 20477; 18, Mixture of *E. durans* DSMZ 20633 and *E. faecium* DSMZ 2146 DNA; B, Blank; M. 1 kb Plus molecular size standard ladder

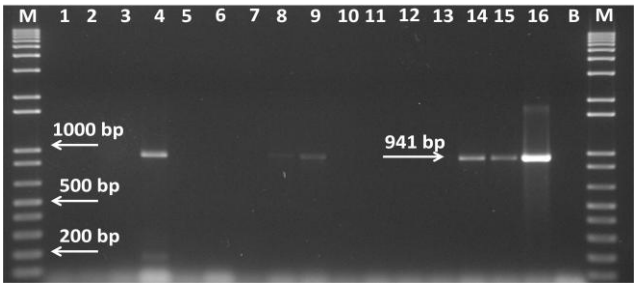

Figure 7 -Example of the species PCR-amplification. Lanes: 1 to 15, meat-enterococci; 15, *E. faecalis* DSMZ 20376; B, Blank; M. 1 kb Plus molecular size standard ladder.

From the total of 148 isolates, three different species were observed, *E. faecalis* (n=78), *E. faecium* (n=59) and *E. durans* (n=11). These results are consistent with the literature (Hugas *et al.*, 2003; Barbosa *et al.*,2009; Ribeiro *et al.*,2011), that described *E. faecalis* and *E. faecium* as the most frequent species found in traditional fermented meat products.

The *E. faecium* isolates were, in the majority, isolated in the SBA supplemented with vancomycin. These results are not unexpected, since the vancomycin resistant phenotype is mostly associated with the *E. faecium* species (Giraffa, 2002).

Dendrograms for each product were also constructed with the PCR-fingerprinting patterns. Figure 8 represents the dendrogram constructed with the 48 isolates obtained from Catalão. This dendrogram was chosen to exemplify the analysis performed for the PCR-fingerprinting.

Above 65% of similarity one can observe the *E. faecalis* cluster (dark blue); the *E. faecium* isolates (dark red) are divided in 3 sub-clusters which group with *E. faecalis* at approximately 55% of similarity. All but one (7C1.5) of the *E. faecium* where isolated from SBA + vancomycin. The diversity index for this dendrogram was of D=0.84, and was calculated with a cutoff line of 70% similarity.

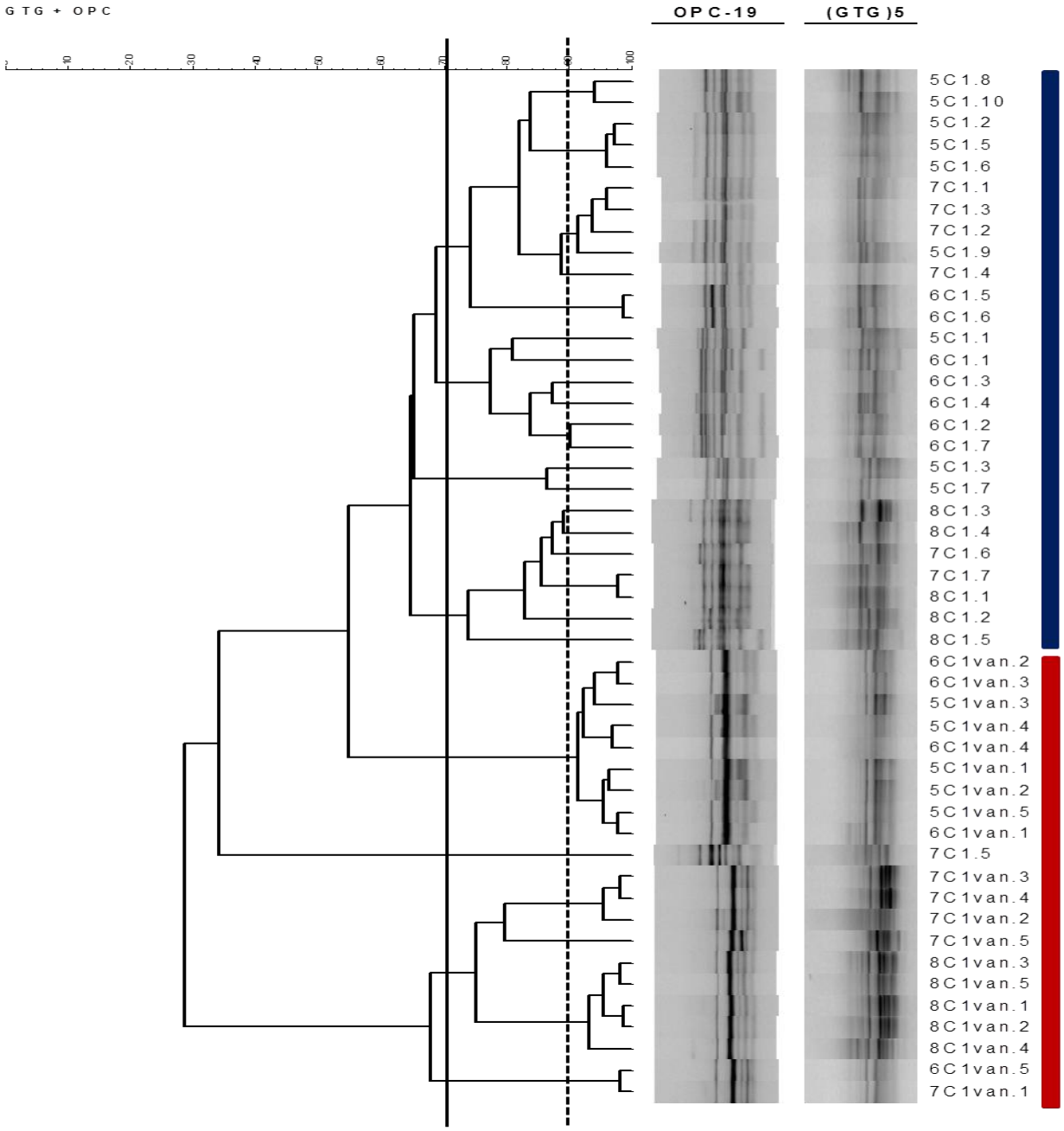

Figure 8 - Dendrogram obtained with the PCR-fingerprinting patterns of the Catalão isolates, grouped by means of Pearson coefficient and the agglomerative clustering of unweighted pair group method with arithmetic mean (UPGMA). Dark blue rectangle: E. faecalis cluster; Dark red rectangle: E. faecium cluster; Dotted line: 90% similarity cutoff level for reproducibility; Full line: 70% similarity cutoff level for the calculation of Simpson's diversity index.

Since the frequency of meat-enterococci was the same for the isolation media supplement and not supplemented with vancomycin, together with the fact that all the vancomycin media isolates are

cluestered in the same group, leads to the conclusion, already proposed by other authors (Cetinkaya *et al.*, 2000), that some VRE are vancomycin dependent. It seems the use of vancomycin was important to select broader enterococcal diversity in the samples studied.

Barbosa *et al.* (2009) and Ribeiro *et al.* (2011) did not isolate any vancomycin resistant enterococci, but did not used vancomycin supplemented media in the isolation step. Comparing with our results, this suggests an underestimation of the vancomycin resistant enterococci.

All the products were positive for the presence of enterococci. Both Chouriço Preto and Linguiça had the highest species diversity, presenting isolates from three different species, *E. faecalis*, *E. faecium* and *E. durans*. In general, all products had high diversity indexes (D > 0.78), being the highest for Catalão, where D=0.84.

3.2. Food technological potential

3.2.1. Bacteriocinogenic properties

Attributed to enterococci is the ability to produce bacteriocins (enterocins). Bacteriocin production may also play an important role in food colonization, especially in dairy or meat fermented products.

The production of a bacteriocin-like substance was evaluated in an agar plate assay. A positive result was considered when a halo effect was observed around the cell-free supernatant spot. This halo-effect reflected the inability of the indicator strains to grow in the presence of the cell-free supernatant.

In this step, the antibactericidal activity was only detected against the *L. monocytogenes* indicator strains. From the six strains, one was isolated from Linguiça, two from Chouriço Preto and three from Salsichão. The presumptive bacteriocin producers were identified as *E. faecalis* (n=4), *E. faecium* (n=1) and *E. durans* (n=1).

Other reports of bacteriocinogenic enterococci present much higher percentages than the observed in the present study. De Vuyst *et al.* (2003) used a very similar approach to screen enterococci from different origins (cheese, animals, clinical and human nonclinical) and found 29% (122/426) *Enterococcus* strains with inhibitory effect against *Listeria*. In the study performed by Vuyst *et al.* (2003), the majority of the bacteriocin producers were identified as *E. faecalis* (68%) and *E. faecium* (59%), as it was in this study.

Following the initial step of identifying putative bacteriocin producers a confirmation of the presence of structural genes was performed for *entA, entB, entL50A/B and entP* by PCR amplification.

The results revealed the presence of two different enterocins, A and B, both present in two different *E. faecalis* strains. These results are not according with De Vuyst *et al.* (2003) which described the presence of structural genes *entA* and *entB* only in *E. faecium* strains.

The structural genes *entL50A/B and entP* were not found in any of the strains. Hence, for the other four strains the structural gene was not identified, where we conclude they do not correspond to any within the group tested. The remaining enterocins may be identified as other frequently produced by enterococci, such as enterocin 31, enterocin 1071, enterocin AS-48, among others.

Nevertheless, the methodology applied does not guarantee that the antimicrobial substances are in deed bacteriocins. To prove so, further testes such as the ones described in Table 8, are still needed. For a bacteriocin, it would be expected the loss of function only in the presence of proteinase K.

Tabela 8 - Bacteriocin confirmation testes.

Treatment	Conditions variations		
Enzymes (1 mg/ml)	**Proteinase K** (37 ºC, 2h)	**Catalase** (37 ºC, 2h)	
Temperature	**60 ºC** (60 min)	**100 º C** (20 min)	**121 ºC** (15 min)
pH	**6**	**7**	**8**

The results obtained point to the potential of meat-enterococci as bacteriocin-producers. However, since they are potential pathogens, their direct use in foods as is not recommended. The alternative would be the use of the purified bacteriocin extract.

3.2.2. Enzymatic activities

As LAB, enterococci have several enzymatic activities attributed that seem to influence the organoleptic traits of traditional fermented products.

Lipase activity was detected with Spirit Blue Agar and Lipase Reagent. A positive result was considered when a clearing beneath and surrounding the colonies was observed, which happened for 2 strains.

Since the fermented meat products under analysis, were fat rich, we expected more lipolytic strains. The inability to produce this enzyme demonstrated by most meat-enterococci could be related with the presence of other bacteria (e.g., Lactobacillus and/or Staphylococcus) responsible for the cleavage of these compounds during the fermentation process. A similar result was observed by Semedo-Lemsaddel *et al.* (2009) and Ribeiro *et al.*, (2011).

Hydrolysis of casein was determined on agar plates containing skimmed milk powder. Caseinolytic activity was considered when clear zones around the spots appeared after incubation of plates, 12 of the tested strains were positive for casein hydrolysis.

The low frequency of hydrolytic enzymes production is consistent with results obtained in other studies with fermented meat-enterococci (Ribeiro *et al.*, 2011).

Only the 6 putative bacteriocin producers strains were screened for enzymatic profiles through the Api-Zym kit (Table 9). The results obtained indicate that each of the isolates produced a different spectrum of enzymes. All of the isolated (6/6) tested positive for the production of cystine arylamidase, acid phosphatase and napthol-AS-BI-phosphohydrolase. On the contrary, none of the isolates (0/6) tested positive for the production of α-galactosidase, β-glucoronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. As for the other enzymes 83% (5/6) of the isolates produced esterase (C4), esterase lipase (C8), leucine arylamidase; 67% (4/6) produced valine arylamidase and α-chymotrypsin; 50% (3/6) produced alkaline phosphatase, trypsin and β-gluconidase; finally 17% (1/6) produced lipase (C14), β-galactosidase and α- gluconidase.

Belgacem *et al.* (2010) also examined the enzymatic profiles of enterococci isolated from fermented meat through the Api-Zym kit and had different results. While some/all of our isolates produced N-acetyl-β-glucosaminidase, alkaline phosphatase, lipase, trypsin, β-galactosidase none of Belgacem's did, but as none of Belgacem's isolates produced α-galactosidase, β-glucoronidase, α-mannosidase and α-fucosidase, so did ours.

These differences seem to indicat that the origin of the isolates definitely determines its enzymatic plasticity, in the matter that the degradation and utilization of different substrates implicate the use of different enzymes. This can also be a colonization advantage by allowing enterococci to degrade a wide variety of substrates and rapidly spread throughout the food.

All the isolates have shown the plasticity usually attributed to enterococci, in the way that all were able to grow in a variety of conditions (at 10ºC, 37º C and 45º C; at presence of 6.5% NaCl; and at pH 9.6). All these phenotypic traits can be usefull in food technology.

Table 9 - Enzymatic profiles of the meat-enterococci**.**

Enzyme/Strain	alkaline phosphatase	esterase (C4)	esterase lipase (C8)	lipase (C14)	leucine arylamidase	valine arylamidase	cystine arylamidase	trypsin	α-chymotrypsin	acid phosphatase	napthol-AS-BI-phosphohydrolase	α-galactosidase	β-galactosidase	β-glucoronidase	α- gluconidase	β-gluconidase	N-acetyl-β-glucosaminidase	α-mannosidase	α-fucosidase
3L1.4	-	+	+	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-
12CP1.3	+	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-
13S2.2	+	+	+	-	-	-	+	-	-	+	+	-	-	-	-	+	-	-	-
15S2.2	-	+	+	-	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-
20P2.1	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-
9CP1van.2	-	-	-	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-

3.3. Pathogenicity potential

3.3.1. Virulence factors

Traditional fermented meat products are largely consumed and have frequently associated the presence of enterococci. This raises the concern to know if these meat-enterococci carry virulence factors. Various virulence factors have been described for enterococci. We investigated the presence of the most common ones.

Hemolysin activity was detected in Colombia Blood Agar, containing 5% of defibrinised horse blood. Zones of clearing around colonies would indicate β-hemolysin production. None of the strains were β-hemolytic. Hemolysin activity is caused by a reaction to erythrocytes and is a very important virulence factor, frequent in clinical enterococci. Its absence among the meat-enterococci indicates a low pathogenicity potential.

Gelatinase activity was tested in a 3% gelatinase medium. A positive result was detected by the formation of a transparent halo. 10 strains produced said halo and were considered positive for gelatinase activity.

Gelatinase was the most frequent enzymatic activity. These results are similar to other previous studys (Eaton and Gasson, 2002; Franz *et al.*, 2001; Semedo-Lemsaddek *et al.*, 2009; Ribeiro *et al.*, 2011). The ability to produce this enzyme, able to hydrolyze gelatin, collagen, casein, and other small biologically active peptides can be explained easily because of the usual presence of such components in the pork meat used to produce traditional fermented meat products.

Several of the most frequent and best described enterococcal virulence factors were screened in this step. This included the coding genes for aggregation substance (*agg*), the *E. faecalis* antigen A (*efaAfs*), the enterococcal surface protein (*esp*), the pili-like (*ebpABC*); gelatinase (*gelE*); gelatinase regulator (*frsB*); cytolysin (*cylA*) and the enterococcal polysaccharide antigen gene (*epa*).

None of the strains was positive for the adhesins genes (both *esp* and *agg*). The lack of aggregation substance, also observed by Ribeiro *et al.* (2011), constitutes an important result, since this protein mediates binding of donor cells to plasmid-free recipients, thus contributing highly to the acquisition and/or dissemination of virulence determinants. Its absence among the meat-enterococci isolates could contribute to their association with low pathogenicity potential.

The *epa* gene was positive in 28% (21/75). The *efaAfs* gene was present at 17% (13/75) of strains. 29% (22/75) of all strains had at least one of the *ebpABC*, but overall the frequency of each gene was different: the *ebpA* gene was detected at 5% (4/75) of the strains, the *ebpB* gene at 19% (14/75) and the *ebpC* gene at 28% (21/75).

In a similar study, Ribeiro *et al.* (2011) reported high incidences of both *ebpABC* and *efaAfs*. The presence of these genes could be advantageous in the manufacturing environment to ensure their maintenance. Nonetheless, our findings seem to demonstrate the opposite.

The *gelE* gene was detected in a total of 75% (56/75) of the strains and the fsrB gene at 25% (19/75) of all strains. Interestingly, the presence of *fsrB* was lower than *gelE*. This phenomenon had been already described by Qin *et al.* (2000).

When *fsrB*, *gelE* and the remaining genes present in the operon, were present we expected a gelatinase activity. Surprisingly, that was not what we observed. That is, only one strain was positive both for *fsrB* and *gelE* and had gelatinase activity; 14 strains were positive both for *fsrB* and *gelE*, but had no gelatinase activity; 2 strains were both negative for *fsrB* and *gelE* and had gelatinase activity; 7 strains were only positive for *gelE* and had gelatinase activity; and no strain was positive only for *fsrB* and had gelatinase activity.

The fact that we observed gelatinase activity in the absence of *fsrB*, but in the presence of *gelE*, can be explained by the fact that the *fsr* locus is composed by more than two genes that can be disrupted, resulting in the absence of gelatinase activity. The same hypothesis can be applied for the cases when both *fsrB* and *gelE* are present but we don't observe gelatinase activity.

Finally, there were two strains that were both negative for *fsrB* and *gelE*. This result can be explained by a false negative in the PCR reaction, which could have failed to detect the presence of *fsrB* and *gelE* genes.

Regarding the secreted virulence factors genes, the *cylA* gene wasn't detected by PCR. Considering that all the genes in the *cyl* operon are needed for a positive hemolytic phenotype, it was no surprise that no strain was β-hemolytic.

The fact that these strains are subjected to a very specific selective pressure in the manufacturing environment may account for the fact that some virulence factors were not detected, i. e. some genes like the *cylA*, *esp and agg*. This may also account for the fact that only four strains were positive for all three *ebp* genes (A, B and C). Some genes could have been lost or even disrupted by movable genetic elements.

Overall, since a low frequency of virulence factors seems to be associated with the meat-enterococci, a low risk is probably associated with the consumption of these fermented meat products.

3.3.2. Susceptibility to antibiotics

The susceptibility to fourteen antibiotics, representing different drug-classes was evaluated. There were four different antibiotics for which we found 100% (75/75) of resistance. They were cefalexin, streptomycin, sulphamethoxazole/trimethoprim and nalidixic acid. On the other hand, none of the isolates were resistant to high level gentamicin. The remaining resistance phenotypes obtained was as follows: 1% (1/75) for ampicillin, 1% (1/75) for amoxycillin/clavulanate, 76% (57/75) for cephotaxim, 3% (2/75) for chloramphenicol, 12% (9/75) for erythromycin, 71% (53/75) for gentamicin-10, 36% (27/75) for penicillin G, 60% (45/75) for tetracycline and 20% (15/75) for vancomycin.

Enterococci are considered intrinsically resistant to β-lactam antibiotics. The results obtained in the present study are not in agreement with this generalization almost all most strains were sensitive to ampicillin, amoxicillin and penicillin. Other authors obtained similar results (Peters *et al.*, 2003; Ben Omar *et al.*, 2004; McGowan-Spicer *et al.*, 2008; Valenzuela *et al.*, 2008; Barbosa *et al.*, 2009). Lopes *et al.* (2005) suggests that resistance of enterococci to β-lactam antibiotics seems to be associated with clinical strains and often inaccurately generalized in the literature to the genus.

Regarding erythromycin, tetracycline, gentamicin, and vancomycin, other studies (Ribeiro *et al.*, 2011), found higher resistance frequencies.

It is important to highlight that from the 19 strains isolated in SB medium supplemented with vancomycin, only 15 had resistant phenotype. More, all the vancomycin resistant strains were isolated using SB medium supplemented with vancomycin, and were present in three different products, five from Linguiça, five from Catalão and six from Chouriço Preto. This observation leads us to believe that the vancomycin resistant phenotype is widespread but underestimated in nature, since some studies did not use the same approach we applied.

The fact that 4 strains isolated using SB medium supplemented with vancomycin, were not vancomycin resistant can be explaind with the fact that the vancomycin concentration, besides not being 100% homogeneious in the plate was also lower than the disc concentration, hence allowing growth of said strains.

For vancomycin resistance strains 5 (out of 15) were found to be high level resistant (MIC> 256 µg/ml). To our knowledge, this study represents the first report of VRE in food products.

According to the Multi-Drug Resistant definition (non-susceptibility to at least one agent in three or more antimicrobial categories) (Magiorakos *et al.*, 2011), all of our strains fell in this category.

The PCR-based detection of the antibiotic resistance genes *erm(B)*, *tet(M)*, *vanA* and *vanB* was tested in the respectively resistant strains.

From the erythromycin resistante strains 78% (7/9) were positive for *erm(B)*, from these *E. faecalis*, and *E. durans*. These results are according to Ribeiro *et al.* (2011) that also found *erm(B)* among erythromycin resistant meat- enterococci.

For tethracyclin resistant strains 65% (26/43) were positive for *tet(M)*, from these *E. faecalis* (n=22), *E. faecium* (n=3) and *E. durans* (n=1). These results are according to other studies (Aarestrup *et al.*, 2000; Del campo *et al.*, 2003; Kuhn *et al.*, 2003; Ribeiro *et al.*, 2011) that observed that *tet(M)* was the most frequently reported tetracycline resistance gene in enterococci.

All of the vancomycin resistant strains were negative either for *vanA* and 5% (1/20) was positive for the *vanB* gene. This strain was identified as *E. faecium*. These results are according to the literature, which

describes the *vanB* genotype to be the most frequent in the European Union, associated with *E. faecium* (Werner, 2011).

The presence of resistant enterococci and the detection of resistance genes amongst the meat-enterococci is a matter of concern, especially when taking into account the level of resistance to vancomycin, which is considered the last resort for treatment of multiple-resistant enterococcal infections.

3.3.3. Enterococcal Biofilms

The results for the detection of biofilm producing enterococci using the microtitre-plate assay are summarized in Table 11. Results show that enterococci can form biofilms on a polystyrene surface. The results obtain allocated the isolates into four groups: non producer or non-biofilm forming ($OD_{590nm} \leq 0.5$); weak producer or weak biofilm forming ($0.5 < OD_{590nm} \leq 1.0$); moderate biofilm forming ($1.0 < OD_{590nm} \leq 1.5$); and strong producer or strong biofilm forming ($1.5 < OD_{590nm} \leq 2.0$).

The non producer's class corresponded to 8% (6/75) of the isolates; the weak producer's class corresponded to 28% (21/75) of the isolates; the medium producer´s class corresponded to 25% (19/75) of the isolates; finally, the strong producer's class corresponded to the biggest percentage of isolates, 39% (29/75).

The control strains *E. faecalis* MMH 594 was included in the strong producer's class with an OD_{590nm} of 2.06.

As expected, *E. faecalis* was the most effective biofilm producer, in general. The majority of the isolates were classified as strong producers. Nonetheless, all the three species identified in this study were classified in the producer's class of biofilm forming enterococci.

Several reports, concerning clinical isolates, indicate that *E. faecalis* produces biofilm more often than *E. faecium* (Mohamed *et al.*, 2004; Rosa *et al.*, 2006; Barbosa *et al.*, 2009).

Table 10 - Biofilm production results.

OD 590nm	Class	Number of strains	Percentage	Percentage/Species
≤ 0.5	Non Producer	6	8%	4% *E.faecalis*
				4% *E. faecium*
]0.5; 1.0]	Weak Producer	21	28%	21% *E. faecalis*
				4% *E. faecium*
				3% *E. durans*
]1.0;1.5]	Moderate Producer	19	25.33%	19% *E. faecalis*
				4% *E. faecium*
				3% *E. durans*
>1.5	Strong Producer	29	38.67%	15% *E. faecalis*
				19% *E. faecium*
				5% *E. durans*
	Total	75	100%	

It's interesting to observe that even though none of the strains were positive for *esp*, the majority felled into a producer class, from weak to strong producer. These results are according to those reported by Kristich *et al.* (2004), were de Esp-independent biofilm formation was described; and by Barbosa *et al.* (2009) for traditional fermented meat products. The same was observed for some biofilm producing strains that lacked the *ebp* genes, including some classified as "Strong Biofilm Producers".

Although more studies are needed, since the majority of the meat-enterococci are biofilm producers, we cannot exclude the possibility that some of the meat-enterococci persist in the manufacturing environment

through biolfilm formation. This is of special concern when we consider the antibiotic resistance profiles and possibility that these bacteria could be responsible for enterococcal infections, particularly among high-risk consumers. To assess this hypothesis a more profound study is needed, one that should involve the analysis of the manufacturing surfaces, products and raw materials, among others.

Population analysis by FISH has proven useful. It can help to understand population structure and biofilm growth over time. In the present work this step consisted in the optimization of a FISH protocol for biofilm forming enterococci. The study was carried out using the *E. faecalis* MMH 594. The tested conditions were: incubation time (24 and 48h); lysozyme concentration for cell permeabilization (0.01, 0.1 and 1 mg/ml); permeabilization time (4 and 10 minutes); cell concentration.

A better cell visualization was observed with a 48 h incubation time. No significant changes were observed when using any of the tested lysozyme concentration, whereby we assumed the lowest concentration to be the better one. The same was observed with the permeabilization time. Since no significant changes were observed with 4 and 10 minutes of permeabilization, we choose 4 minutes. Considering the initial cell concentration of 10^8 CFU/g, we found useful to use decimal dilution to observe the biofilm forming populations. Since the initial step of protocol optimization was overcome, the next step would be to apply it to the meat-enterococci that have shown biofilm production ability.

Enterococci have a duality associated due to the fact that they are both comensal organisms and typically harbor virulence factors and antibiotic resistances. The Mediterranean fermented meat products have a high frequency of enterococci associated. Luckly, there has been no report of an enterococcal infection directly associated with the consumption of food product harboring these bacteria.

The meat-enterococi analyzed in these study, have shown a technological potential (growth at 10-45ºC, pH 9.6, 40% NaCl, bacteriocin production, broad enzymatic activity). Althought they are bacteriocin producers and have a broad range of enzymatic activities, at the same time they have associated high-level of antiobiotic resistance. This could endanger their use in food. An interesting example is the strain 9CP1van.2, which represent an extreme. On one hand, it is bacteriocin producer and has a broad enzymatic activity. On the other hand, it's high level vancomyin resistant and moderate biofilm producer.

It is interesting to observe that the 6 strains with food-techonogical potential also have associated resistance to several antibiotics, presence of several virulence factors and in some cases are even biofilm producer. The most interesting case is the 9CP1van.2 that is VRE, *vanB*$^+$ and biofilm producer. These observations validate the delicate nature of using enterococci as added cultures in food products.

Overall, since a low number of virulence factors were observed, a low risk seems to be associated with the presence of enterococci in these long-established traditional meat fermented products.

4. Conclusions:

The present inveswtigation tried to answers questions regarding the underlying duality of food-enterococci.

The first aim of this study was to evaluate the presence of enterococci in Portuguese traditional fermented meat products. We observed that enterococci are well established in these products. Enterococci were found in all foodstuffs at a frequency of 10^4 CFU/g for Catalão, Chouriço Preto and Linguiça. As for the Salsichão and Paio the frequency was lower, being less than 10^2 CFU/g.

For the presumptive genus identification all isolates were tested for the ovoid cell morphology, Gram-positive, catalase and oxidase negative, and growth at 10ºC, 37º C and 45º C, at presence of 6.5% NaCl and at pH 9.6. a definitive genus identification was obtained with the PCR-amplification of the *tuf* gene. A total of 148 isolates were identifies as *Enterococcus* spp.

Secondly, we wanted to assess which were the main species present in these products. In the species level identification step we were able to confirmation the presence of three different species, *E. faecalis* (n=78), *E. faecium* (n=59) and *E. durans* (n=11). The predominant species in all products were *E. faecalis* and *E. faecium*. In the isolation medium supplemented with vancomycin, all but one species was *E. faecium*.

Subsequently, we wanted to investigate the diversity of the bacterial collection through the use of a PCR-fingerprinting technique, with primers OPC-19 and $(GTG)_5$ and calculation of Simpson's diversity index (D=0.927). Analysis of the dendrogram obtained from the PCR-fingerprinting patterns led to the selection of 75 meat-enterococci, representative isolates of all enterococcal groups, for further studies.

For each product a dendrogram was constructed with the PCR-fingerprinting patterns obtained for the meat-enterococci. In all cases, very distinct clusters formed, corresponding to the different species.

We also wanted to assess for the technological potential of the isolates. To do so, we wanted to understand if meat-enterococci could be bacteriocin producers and determine the main antimicrobial spectrum of action of said bacteriocin(s). To answer this question, we screened the meat-enterococci for bacteriocin production against five pathogens that included *L. monocytogenes*, *Escherichia. coli*, *Salmonella enterica* serovar Enteritidis and vancomycin resistant *E. faecium* and *E. faecalis*.

The bacteriocinogenic potential of the meat-enterococci seemed to be much reduced, since only 6 strains out of 75 had putative antimicrobial activity against *L. monocytogenes*. It was also important to understand which bacteriocin(s) they produced. We were able to identify the structural genes *entA* and *entB* in two of the strains, while the other remained unidentified.

Also regarding the technological potential of the meat-enterococci we investigated which enzymatic activities do meat-enterococci posses. Concerning the hydrolytic enzymes production; 10 strains were positive for gelatinase activity, 2 strains were positive for lipase activity and 12 for casein hydrolysis. The 8% enterococci that produced bacteriocins also had several enzymatic activities. All were tested positive for the production of cystine arylamidase, acid phosphatase and napthol-AS-BI-phosphohydrolase.

Regarding virulence we aimed to investigate which virulence factors meat-enterococci have. Through a PCR-amplification method, we found that the most frequent were *gelE* (75%) and *ebpC* (28%). the virulence factors *agg*, *esp* and *cylA* were absent.

We also screened for antibiotic suceptibility and found all isolates to be resistant to streptomycin, cephalexin, nalidixic acid and trimethropim/sulfamethoxazole; and 99% of the isolates to be susceptible to

gentamicin high level, ampicilin, amoxicillin and chloramphenicol. All the strains under analysis were multi-drug resistant.

All VRE (20%) were isolated from medium supplemented with vancomycin. From these, 5 were found to also be high level vancomycin resistant (>256 µg/ml).

Our final aim, was to understand if the meat-enterococci could produce biofilms that may allow them to persist in the manufacturing environment. Biofilm production was assessed for the enterococci in study, 6% of the strains were classified as nonbiofilm producer. On the contrary, the majority was classified as strong biofilm producer (39%).

Within the biofilm producers, the most comon species were *E. feacalis*, followed by *E. faecium*. None *E. durans* strain was classified as nonbiofilm producer.

The meat-enterococci under study seem to have a high level of resistance to antibiotics, but due to the low number of virulence factors observed, a low risk appears to be associated with the consumption of these products. This evidence is supported by the fact that there are no reports of toxinfection by enterococci connected with the consumption of traditional Portuguese fermented meat products.

Even though, some enterococal strains showed technological potential, our study allowed to percieve underlying antibiotic resistances and presence of virulence factors, that descart the direct use of these strains in food technology.

5. References:

Aarestrup F. M., Seyfarth A. M., Emborg H. D., Pedersen K., Hendriksen R., Bager F. (2001) Effect of Abolishment of the Use of Antimicrobial Agents for Growth Promotion on Occurrence of Antimicrobial Resistance in Fecal Enterococci from Food Animals in Denmark. *Antimicrobial Agents and Chemotherapy*, 45(7): 2054-2059.

Amann R. I., Binder B. J., Olson R. J., Chisholm S. W., Devereux R., Stahl D. A. (1990) Combination of 16S rRNA-Targeted Oligonucleotide Probes with Flow Cytometry for Analyzing Mixed Microbial Populations. *Applied and Environmental Microbiology*, 56(6): 1919-1925.

Aminov R. I., Garrigues-Jeanjean, Mackie R. I. (2001) Molecular Ecology of Tetracycline Resistance: Development and Validation of Primers for Detection of Tetracycline Resistance Genes Encoding Ribosomal Protection Proteins. Applied and Environmental Microbiology, 67(1): 22-32.

Anonymous (2000) Qualité de l'eau. Recherche et dénombrement des entérocoques intestinaux – Partie 2: Méthode par filtration sur membrane - NF EN ISO 7899-2.

Anonymous (2010) Annual epidemiological report on communicable diseases in Europe. Surveillance Report. European Centre for Disease Prevention and Control. Annual. Stockholm: ECDC ISBN 978-92-9193-222-1

Alves P. I., Martins M. P., Semedo T., Marques J. J. F., Tenreiro R., Crespo M. T. B. (2004) Comparison of phenotypic and genotypic taxonomic methods for the identification of dairy enterococci. *Antonie van Leeuwenhoek* 85: 237–252.

Arias C. A., Robredo B., Singh K. V., Torres C., Panesso D., Murray B. E. (2006) Rapid Identification of *Enterococcus hirae* and *Enterococcus durans* by PCR and Detection of a Homologue of the *E. hirae mur-2* Gene in *E. durans*. *Journal of Clinical Microbiology*. 44(4): 1567–1570.

Barbosa J., Ferreira V., Teixeira P. (2009) Antibiotic susceptibility of enterococci isolated from traditional fermented meat products. *Food Microbiology*, 26:527-532.

Ben Omar N., Castro A., Lucas R., Abriouel H., Yousif N. M. K., Franz C. M. A. P., Holzapfel W. H., Rubén P. P., Marínez-Canamero M., Gálvez A. (2004) Functional and Safety Aspects of Enterococci Isolated from Different Spanish Foods, *Systematic and Applied Microbiology*, 27(1):118-130.

Bevilacqua A., Altieri C., Corbo M. R., Sinigaglia M., Ouba L. I. I. (2010) Characterization of Lactic Acid Bacteria Isolated from Italian Bella di Cerignola Table Olives: Selection of Potential Multifunctional Starter Cultures. *Journal of Food Science*, 75(8):536-544.

Borgen K., Sorum M., Wasteson Y., Kruse H., Oppegaard H. (2002) Genetic linkage between *erm*(B) and *vanA* in *Enterococcus hirae* of poultry origin. *Microbial Drug Resistance*, 8:363-368.

Carlos, A. R., Semedo-Lemsaddek, T., Barreto-Crespo, M. T., & Tenreiro, R. (2010). Transcriptional analysis of virulence-related genes in enterococci from distinct origins. *Journal of Applied Microbiology*, 108(5):1563-1575.

Cetinkaya Y., Falk P., Mayhall C. G. (2000) Vancomycin-Resistant Enterococci. *Clinical Microbiology Reviews*, 13(4):689-707.

Chow, J., Thal L., Perri M., Vazquez J., Donabedian S., Clewell D., Zervos M. (1993) Plasmid-associated hemolysin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. *Antimicrobial Agents and Chemotherapy,* 37: 2474–2477.

Clewell D. B. (1990) Movable Genetic Elements and Antibiotic Resistance in Enterococci. *European Journal of Clinical Microbiology & Infectious Diseases*, 9(2):90-102.

Coque T. M., Freitas A. R., Novais C., Peixe L., Baquero F. (2011) Mobile Genetic Elements and Lateral Genetic Transferi n Enterococci, in Semedo-Lemsaddek T. Enterococcus and Safety, Nova Science Publishers, Inc.

Costerton J.W., Stewart P. S., Greenberg E. P. (1999) Bacterial Biofilmes: A common cause of persistent infections. *Science*, 284: 1318-1322.

Crespo M. T. B., Alves P. I. (2011) Enterococci in Food. . In: Semedo-Lemsaddek T., Barreto-Crespo M. T., Tenreiro R. (Ed.) *Enterococcus* and Safety, Nova Science Publisher, New York, In Press.

Del Campo R., Ruiz-Garbojasa P. R., Sánchez-Moreno M. P., Baquero F., Torres C., Cantón R., Coque T. M. (2003) Antimicrobial Resistance in Recent Fecal Enterococci from Healthy Volunteers and Food Handlers in Spain: Genes and Phenotypes. *Microbial Drug Resistance*, 9(1):47-60.

Devriese L. A. Pot B., Collins M. D. (1993) Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. *Journal of Applied Bacteriology*, 75:399-408.

De Vuyst L., Moreno M. R. F., Revets H. (2003) Screening for enterocins and detection of hemolysin and vancomycin resistance in enterococci of different origins. *International Journal of Food Microbiology*, 84: 299-318.

Dunny G. M. (1990) Genetric functions and cell-cell interactions in the pheromone-inducible plasmid transfer system of *Enterococcus faecalis*. *Plasmid*, 4: 689-696.

Eaton T. J., Gasson M. J. (2001) Molecular Screening of *Enterococcus* Virulence Determinants and Potential for Genetic Exchange between Food and Medical Isolates. *Applied and Environmental Microbiology*. 67(4): 1628–1635.

Eaton T. J., Gasson M. J. (2002) A variant enterococcal surface protein ESPfm in *Enterococcus faecium*; distribution among food, commensal, medical, and environmental isolates. *FEMS Microbiology Letters*, 216: 269-275.

Elsayed S., Hamilton N. (2001) Improved Primer Design for Multiplex PCR Analysis of Vancomycin-Resistant *Enterococcus* spp., *Journal of Clinical Microbiology*, 39(6): 2367–2368.

Extremina C. I., Costa L., Aguiar A. I., Peixe L., Fonseca A. P. (2010) Optimization of processing conditions for the quantification of enterococci biofilms using microtitre-plates. *Journal of Microbiological Methods*, 84(2):167-173

Franz C. M. A. P., Holzapfel W. H., Stiles M. E. (1999) Enterococci at the crossroads of food safety? *International Journal of Food Microbiology*, 47: 1-24.

Franz C. M. A. P., Stiles M. E., Schleifer K. H., Holzapfel W. H. (2003) Enterococci in foods—a conundrum for food safety, *International Journal of Food Microbiology* 88: 105– 122.

Franz C. M. A. P., Belkum M. J. van, Holzapfel W., Abriouel H., Galvez A. (2007) Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. *FEMS Microbiology Reviews*, 31: 293-310.

Franz C. M. A. P., Muscholl-Silberhorn A. B., Yousif N. M. K., Vancanneyt M., Swings J, Holzapfel W. H. (2001) Incidence of Virulence Factors and Antibiotic Resistance among Enterococci Isolated from Food. *Applied and Environmental Microbiology*, 67(9):4385-4389.

Gilmore M. S., Segarra R. A., Booth M. C., Bogie C. P., Hall L. R., Clewell D. B. (1994) Genetic Structure of the *Enterococcus faecalis* Plasmid pADi- Encoded Cytolytic Toxin System and Its Relationship to Lantibiotic Determinants, *Journal of Bacteriology*, 176(23): 7335-7344.

Giménez-Pereira, Lorena M. (2005) Enterococci in milk products : a dissertation presented in partial fulfilment of the requirements for the degree of Master of Veterinary Studies at Massey University, Palmerston North, New Zealand

Giraffa G. (2002) Enterococci from foods. FEMS *Microbiology Reviews*, 26: 163-171.

Haas W., Gilmore M. S. (1999) Molecular nature of a novel bacterial toxin: the cytolysin of *Enterococcus faecalis*. *Medical Microbiology and Immunology*. 187:183–190.

Heikens E., Bonten M., Willems R. J. (2007) Enterococcal Surface Protein Esp is important for Biofilm formation of *Enterococcus faecium* E1162. *Journal of Bacteriology*, 189(22): 8233-8240.

Hsieh S. E., Hsu L. L., Hsu W. H., Chen C. Y., Chen H. J., Liao C. T. (2006) Importance of amino acid alterations and expression of penicillin-binding protein 5 to ampicillin resistance of *Enterococcus faecium* in Taiwan. *International Journal of Antimicrobial Agents*, 29:514-519.

Horodniceanu T, Bougueleret L, El-Solh N, Bieth G, Delbos F. (1979) High-level, plasmid-borne resistance to gentamicin in *Streptococcus faecalis* subsp. zymogenes. *Antimicrob Agents Chemother*, 16:686-689.

Hugas M., Garriga M., Aymerich M. T. (2003) Functionalty of enterococci in meat products, *International Journal of Food Microbiology* 88: 223– 233.

Hunter P. R., Gaston M. A. (1988) Numerical index of the discriminatory ability of typing schemes: an application of Simpson's index of diversity. *Journal of Clinical Microbiology*. 26: 2465-2466.

Ike Y., Clewell D. B. (1992) Evidence that the Hemolysin/Bacteriocin Phenotype of *Enterococcus faecalis* subsp. *zymogenes* Can Be Determined by Plasmids in Different Incompatibility Groups as Well as by the Chromosome. *Journal of Bacteriology*, 174(24): 8172-8177.

Jacoby G. A. (2005) Mechanisms of resistance to quinolones. *Clinical Infectious Diseases* , 41 Suppl 2:S120-S126.

Jackson C. R., Fedorka-Cray P. J, Barrett J. B. (2004) Use of a Genus- and Species-Specific Multiplex PCR for Identification of Enterococci. *Journal of Clinical Microbiology*. 42(8): 3558–3565.

Jackson C. R., Fedorka-Cray P. J, Barrett J. B., Ladelu S. R. (2004) Genetic relatedness of high-level aminoglycoside-resistant enterococci isolated from poultry carcasses. *Avian Diseases*, 48(1):100-107.

Jett B. D., Huycke M. M., Gilmore M. S. (1994) Virulence of enterococci. *Clinical Microbiology Reviews*, 7(4): 462-478.

Jurkovič D., Križková L., Dušinský R., Belicová A., Sojka M., Krajčovič J., Ebringer L. (2006) Identification and characterization of enterococci from bryndza cheese. *Letters in Applied Microbiology*. ISSN 0266-8254

Ke D., Picard F. J., Martineau F., Ménard C., Roy P. H., Oullette M., Bergeron M. G. (1999) Development of a PCR Assay for Rapid Detection of Enterococci. *Journal of Clinical Microbiology* 37(11): 3497–3503.

Klaenhammer T. R. (1988) Bacteriocins of lactic acid bacteria. *Biochemie*, 70: 337-349.

Klein G. (2003) Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *International Journal of Food Microbiology* 88: 123-131.

Kristich C. J., Li Y. H., Cvitkovitch D. G., Dunny G. M. (2004) Esp- independent biofilm formation by *Enterococcus faecalis*. *Journal of Bacteriology*, 186: 154-163.

Kuhn I., Iversen A., Burman L. G., Olsson-Lijequist B., Franklin A., Finn M., Aarestrup F., Seyfarth A. M., Blanch A. R., Vilanova X., Taylor H., Caplin J., Moreno M. A., Dominguez L., Herrero I. A., Mollby R. (2003) Antimicrobial Resistance in Recent Fecal Enterococci from Healthy Volunteers and Food Handlers in Spain: Genes and Phenotypes. *International Journal of Food Microbiology*, 88(2-3):133-145.

Leclerq R., Derlot E., Weber M., Duval J., Courvalin P. (1989) Transferable Vancomycin and Teicoplanin Resistance in *Enterococcus faecium*. *Antimicrobial Agents and Chemotherapy*, 33(1): 10-15.

Lopes M. F. S., Simões A. P., Tenreiro R., Marques J. J. F., Crespo M. T. B. (2006) Activity and expression of a virulence factor, gelatinase, in dairy enterococci. *International Journal of Food Microbiology*, 112: 208-214.

Lowe A. M., Lambert P. A., Smith A. W. (1995) Cloning of an *Enterococcus faecalis* Endocarditis Antigen: Homology with Adhesins from Some Oral Streptococci. *Infection and Immunity*, 63(2): 703-706.

Maietti L., Bonvini B., Huys G., Giraffa G. (2007) Incidence of antibiotic resistance and virulence determinants among Enterococcus italicus isolates from dairy products. *Systematic and Applied Microbiology*, 30:509-517.

Magiorakos A. P., Srinivasan A. Carey R. B., Carmeli Y., Falagas M. E., Giske C. G., Harbarth S., Hindler J. F., Kahlmeter G., Olssom-Lijequist B., Paterson D., Rice L. B., Stelling J., Struelens M. J., Vatopoulos A., Weber J. T., Monnet D. L. (2011) Clinical Microbiology and Infection, Published online doi: 10.1111/j.1469-0691.2011.03570.x.

Mannu L., Paba A., Daga E., Comunian R., Zanetti S., Dupré I., Sechi L.A. (2003) Comparison of the incidence of virulence determinants and antibiotic resistance between Enterococcus faecium strains of dairy,animal and clinical origin. *International Journal of Food Microbiology*, 88:291-304.

Mahbub A. M., Kobayashi N., Ishino M., Sumi A., Kobayashi K., Uehara N., Watanabe N. (2005) Detection of a novel *aph*(2") allele (*aph*[2"]-Ie) conferring high-level gentamicin resistance and a spectinomycin resistance gene *ant*(9)-Ia (*aad* 9) in clinical isolates of enterococci. *Microbial Drug Resistance* , 11:239-247.

Macovei L., Zurek L. (2006) Ecology of Antibiotic Resistance Genes: Characterization of Enterococci from Houseflies Collected in Food Settings. *Applied and Environmental Microbiology*, 72(6): 4028–4035.

McGowan-Spicer, Lori L, Fedorka-Cray, Paula J., Frye, Jonathan G., Meinersmann, Richard J., Barrett, John B., Jackson, Charlene R. (2008) Antimicrobial Resistance and Virulence of *Enterococcus faecalis* Isolated from Retail Food, *Journal of Food Protection* 71(4):760-769.

Mohamed J. A., Huang D. B. (2007) Biofilm formation by enterococci. *Journal of Medical Microbiology*, 56: 1581-1588.

Mohamed J. A., Huang W., Nallapareddy S. R., Teng F. Murray B. E. (2004) Influence of Origin of Isolates, Especially Endocarditis Isolates, and Various Genes on Biofilm Formation by *Enterococcus faecalis*. *Infection and Immunity*, 72(6): 3658-3663.

Murray B. E. (1990) The Life and Times of the Enterococcus. Clinical Microbiology Reviews, 3(1):4 6-65.

Nes I. F., Diep D. B., Håvarstein L. S., Brurberg M. B., Eijsink E., Holo H. (1996) Biosynthesis of

Noble W. C. (1992) Co-transfer of vancomycin and other resistance genes from Enterococcus faecalis NCTC 12201 to Staphylococcus aureus. *FEMS Microbiology Letters*, 2(1): 195-198.

Ogier J. C., Serror P. (2008) Safety assessment of dairy microorganisms: The Enterococcus genus. *International Journal of Food Microbiology*, 126: 291-300.

Patel R., Piper K. E., Rouse M. S., Steckelberg J. M. Uhl J. R., Kohner P., Hopkins M. K., Cockerill F. R., Kline B. C. (1998) Determination of 16S rRNA sequences of enterococci and application to species identification of nonmotile *Enterococcus gallinarum* infections. *Journal of Clinical Microbiology*, 36:3399-3407.

Paganelli F. L., Leavis H. L. (2011) Enterococcal Biofilms. In: Semedo-Lemsaddek T., Barreto-Crespo M. T., Tenreiro R. (Ed.) *Enterococcus* and Safety, Nova Science Publisher, New York, In Press.

Pangallo D., Harichová J., Karelová E., Drahovská H., Chovanová K., Ferianc P., Turňa J., Timko J. (2004) Molecular investigation of enterococci isolated from different environmental sources. *Biologia*, 59(6): 829-837.

Peters J., Mac K., Wichmann-Schauer H., Klein G., Ellerbroek L. (2003) Species distribution and antibiotic resistance patterns of enterococci isolated from food of animal origin in Germany. *International Journal of Food Microbiology*, 88(2-3):311-314.

Qin X., Singh K. V., Weinstock G. M., Murray B. E. (2000) Effects of Enterococcus faecalis fsr genes on production of gelatinase and serine protease and virulence. *Infection and immunity*, 68(5): 2579-2586.

Qin X., Singh K. V., Weinstock G. M., Murray B. E. (2001) Characterization of *fsr*, a Regulator Controlling Expression of Gelatinase and Serine Protease in *Enterococcus faecalis* OG1RF. *Journal of Bacteriology*, 183(11): 3372-3382.

Ribeiro T., Oliveira M., Fraqueza M. J., Lauková A., Elias M., Tenreiro R., Barreto A. S., Semedo-Lemsaddek T. (2011) Antibiotic Resistance and Virulence Factors among Enterococci Isolated from Chouriço, a Traditional Portuguese Dry Fermented Sausage. *Journal of Food Protection* 74(3):465-469.

Rice L. B., Bellais S., Carias L. L., Hutton-Thomas R., Bonomo R. A., Caspers P., Page M. G. P., Gutmann L. (2004) Impact of Specific *pbp5* Mutations on Expression of β-Lactam Resistance in *Enterococcus faecium*. *Antimicrobial Agents and Chemotherapy*, 48(8): 3028-3032.

Roberts M. C. (2005) Update on acquired tetracycline resistance genes. *FEMS Microbiology Letters*, 245:195-203.

Roberts M. C., Sutcliffe J., Courvalin P., Jensen L. B., Rood J., Seppala H. (1999) Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. *Antimicrobial Agents Chemother*, 43:2823-2830.

Rosa R. D., Creti R. Venditti M., D'Amelio R., Ariciola C. R., Montanaro L., Baldassarri L. (2006) Relationship between bio¢lmformation, the enterococcal surface protein (Esp) and gelatinase in clinical isolates of *Enterococcus faecalis* and *Enterococcus faecium*. *FEMS Microbiology Letters*, 256: 145-150.

Semedo T., Santos M. A., Lopes M. F. S., Marques J. J. F., Crespo M. T. B., Tenreiro R. (2003) Virulence Factors in Food, Clinical and Reference Enterococci: A Common Trait in the Genus?, *Systematic Applied Microbiology*, 26: 13-22.

Semedo-Lemsaddek T, Mato R. (2011) Pathogenesis and Virulence. In: Semedo-Lemsaddek T., Barreto-Crespo M. T., Tenreiro R. (Ed.) *Enterococcus* and Safety, Nova Science Publisher, New York, In Press.

Semedo-Lemsaddek T., Tenreiro R., Alves P. L., Barreto-Crespo M. T. (2009) Enterococcus 157-179. I*n:* Liu D. (Eds) *Molecular Detection of Foodborn Pathogens.* CRC Press*.*

Singh K. V., Coque T. M., Weinstock G. M., Murray B. E. (1998) In vivo testing of an *Enterococcus faecalis efaA* mutant and use of efaA homologs for species identication. *FEMS Immunology and Medical Microbiology*, 21:323-331.

Sistek V., Maheux A. F., Boissinot M., Bernard K. A., Cantin P., Cleenwerck I., De Vos P., Bergeron M. G. (2011) Two novel species, *Enterococcus ureasiticus* sp. nov. and *Enterococcus quebecensis* sp. nov., isolated from water. International Journal os Systematic and Evolutionary Microbiology, Published online doi: 10.1099/ijs.0.029033-0.

Stiles M. E., Holzapfel W. H. (1997) Lactic acid bacteria and their current taxonomy. I*nternational Journal of Food Microbiology*, 36: 1-29.

Švec P., Vancanneyt, Seman M., Snauwaert C., Lefebvre K. Sedláček I., Swings J. (2005) Evaluation of $(GTG)_5$-PCR for identification of *Enterococcus* Spp., *Microbiology Letters*, 247:59-63.

Tendolkar P. M., Baghadayan A. S., Gilmore M. S., Shankar N. (2004) Enterococcal surface Protein, Esp, Enhances Biofilms Formation by *Enterococcus faecalis*. *Infection and Immunity*,72(10): 6032-6039.

Todd E. W. (1934) A comparative serological study of streptoloysins derived from human and from animal infections, with notes on pneumococcal haemolysin, tetanolysin and staphylococcus toxin. *Journal of Pathology and Bacteriology*, 39: 299-321.

Toledo-Arana A., Valle J., Solano C., Arrizubieta M. J., Cucarella C., Lamata M., Amorena B., Leiva J., Penadés J. R., Lasa I. (2001) The Enterococcal Surface Protein, Esp, is involved in *Enterococcus faecalis* Biofilm Formation. *Applied and Environmental Microbiology*, 67(10): 4538-4545.

Valenzuela A. S., Bem Omar N., Abriouel H., López R. L., Veljovic K., Canamero M. M., Topisirovic M. K. L., Gálvez A. (2009) Virulence factors, antibiotic resistance, and bacteriocins in enterococci from artisan foods of animal origin. *Food Control*, 20:381-385.

Yean C. Y., Yin L. S., Lalitha P., Ravichandran M. (2007) A nanoplex PCR assay for the rapid detection of vancomycin and bifunctional aminoglycoside resistance genes in *Enterococcus* species. BioMed Central Microbiology, 7(112):1-8.

Wegener H. C., Aerestrups F. M., Jensen L. B., Hammerum A. M., Bager F. (1999) Use of Antimicrobial Growth Promoters in Food Animals and Enterococcus faecium Resistance to Therapeutic Antimicrobial Drugs in Europe. *Emerging Infectious Diseases*, 5(3):329-335.

Werner G. (2011) Surveillance of antimicrobial resistance among *enterococcus faecium* and *enterococcus faecalis* isolated from human (clinical/commensal), food animal, meat and environmental samples. In: Semedo-Lemsaddek T., Barreto-Crespo M. T., Tenreiro R. (Ed.) *Enterococcus* and Safety, Nova Science Publisher, New York, In Press.

Wirth R. (1994) The sex pheromone system of *Enterococcus faecalis*. More than just a plasmid-collection mechanism? *European Journal of Biochemistry*, 222: 235–246.

Appendix A

Primers List

	Primer	Amplicon size (bp)	Control Strains	Target species	Gene (locus)	Sequence (5'-3')	PCR optimized conditions	Ref.
Genus Identification	Ent1 Ent2	112	*E. faecium 2146^T and 20477^T, E. faecalis 2037 and 20478^T, E. hirae 20160^T, E. durans 20633^T, and E. casseliflavus 20680^T*	*Enterococcus spp.*	*Tuf* gene (elongation factor EF-Tu)	5'-TACTGACAAACCATTCATGATG-3' 5'-AACTTCGTCACCAACGCGAAC-3'	95 ºC (5 min), 35 cycles of 95 ºC (1 min), 48 ºC (1 min), 72 ºC (1 min) 72 ºC (10 min) 4 ºC (infinite)	Ke *et al.*, 1999
Fingerprinting	$(GTG)_5$	]200-3000[	*E. faecium 2146^T and 20477^T, E. faecalis 2037 and 20478^T, E. hirae 20160^T, E. durans 20633^T, and E. casseliflavus 20680^T*	n.a	Microsatellite	5'-GTGGTGGTGGTGGTG-3'	95 ºC (5 min), 40 cycles of 95 ºC (1 min), 40 ºC (1 min), 72 ºC (1 min) 72 ºC (10 min) 4 ºC (infinite)	Švec *et al.,* (2005)
	OPC19	]200-3000[			Random sequence	5' - GTTGCCAGCC - 3'		Not published
Species Identification	ddlE1 ddlE2	941	*E. faecalis* 2037 and 20478^T	*E. faecalis*	$ddl_{Ent.faecalis}$ (chromosomally encoded D-Ala:D-Ala ligase)	5' – ATCAAGTACAGTTAGTCTT – 3' 5' - ACGATTCAAAGCTAACTG – 3'	95 ºC (5 min), 35 cycles of 95 ºC (1 min), anneling temperatura (1 min), 72 ºC (1 min) 72 ºC (10 min) 4 ºC (infinite)	Jurkovic *et al.*, (2006)
Species Identification	FM1 FM2	215			*sodA* (superoxide dismutase gene)	5' – GAAAAAACAATAGAAGAATTAT – 3' 5' - TGCTTTTTTGAATTCTTCTTTA – 3'		Jackson *et al.*, (2004)

Species Identification	ddlF1 ddlF2	550	*E. faecium* 2146^{T} and 20477^{T},	*E. faecium*	*ddl*$_{Ent.faecium}$ (chromosomally encoded D-Ala:D-Ala ligase)	5’ – GCAAGGCTTCTTAGAGA – 3’ 5’ - CATCGTGTAAGCTAACTTC – 3’		Arias *et al.*, 2006
Species Identification	FL1 FL2	360			*sodA* (superoxide dismutase gene)	5’ – ACTTATGTGACTAACTTAACC – 3’ 5’ - TAATGGTGAATCTTGGTTTGG – 3’		Jackson *et al.*, (2004)
Species Identification	DU1 DU2	295	*E. durans* 20633^{T}	*E. durans*	*sodA* (superoxide dismutase gene)	5’ – CCTACTGATATTAAGACAGCG – 3’ 5’ – TADSMZTAAGATAGGTGTTTG – 3’		Jackson *et al.*, (2004)
Species Identification	mur-2-F mur-2-R	521	*E. casseliflavus* 20680^{T}	*E. casseliflavus*	*mur-2* muramidase gene	5’ – CGTCAGTACCCTTCTTTTGCAGAGTC – 3’ 5’ - GCATTATTACCAGTGTTAGTGGTTG – 3’		Arias *et al.*, 2006
Species Identification	CA1 CA2	288			*sodA* (superoxide dismutase gene)	5’ – TCCTGAATTAGGTGAAAAAAC – 3’ 5’ - GCTAGTTTACCGTCTTTAACG – 3’		Jackson *et al.* (2004)
ITS-PCR	pS1490 pL132	various	*E. faecium 2146^{T} and 20477^{T}, E. faecalis 2037 and 20478^{T}, E. hirae 20160^{T}, E. durans 20633^{T}, and E. casseliflavus 20680^{T}*	*Enterococcus* spp.	Intergenic spacer regions	5’-TGCGGCTGG DSMZCCTCCTT-3’ 5’- CCGGGTTTCCCCATTCGG-3’	95 ºC (5 min), 35 cycles of 95 ºC (1 min), 48 ºC (1 min), 72 ºC (1 min) 72 ºC (10 min) 4 ºC (infinite)	Alves *et al.*, (2004)
Bacteriocin Production	entA	294	AV25a	n.a	Enterocin A	5’- TTAGGTGGAGCAATTCCAGG – 3’ 5’ – CCAGCAGTTCTTCCAATTTCA – 3’	95 ºC (3 min), 30 cycles of 95 ºC (30 seg), 55 ºC (30 seg), 72 ºC (30 seg) 72 ºC (5 min) 4 ºC (infinite)	Pangallo *et al.* (2004)

Bacteriocin Production	entB	151	AV25a	n.a	Enterocin B	5’ – CGAAAGAGATGAAACAAATTATCG – 3’ 5’ – CATGCTAGTGGTCCTTTTGGG – 3’		Pangallo *et al.* (2004)
Bacteriocin Production	entL50A /B	237	AS34b	n.a	Enterocin L50A/B	5’ – GGAGCAATCGCAAAATTAGTAG -3’ 5’ – TGTCCAATAAATTGCATGATTTG - 3’		Pangallo *et al.* (2004)
Bacteriocin Production	entP	206	AS34b	n.a	Enterocin P	5’ – GATGCAGCTACGCGTTCATA – 3’ 5’ – GGGTGGCTAATGCTGTTTCA – 3’		Pangallo *et al.* (2004)
Virulence factor	*esp*	933	*E. faecalis* MMH 594	*Enterococcus* spp.	enterococcal surface protein	5’ – TTGCTAATGCTAGTCCACGACC – 3’ 5’ – GCGTCAACATTGCATTGCCGAA – 3’	95 ºC (3 min), 30 cycles of 95 ºC (30 seg), 55 ºC (30 seg), 72 ºC (30 seg) 72 ºC (5 min) 4 ºC (infinite)	Eaton and Gasson, 2001
Virulence factor	*ebpA*	744	*E. faecalis* MMH 594	*Enterococcus* spp.	endocarditis/biofilm-associated pilus	5’ – TCCATTTGCAGAAGCAAGAATG -3’ 5’ – CCDSMZGCCTCTGTTTGAACTC - 3’		Personal comunication
Virulence factor	*ebpB*	238	*E. faecalis* MMH 594	*Enterococcus* spp.		5’ – GCATTAGCAGAGGCATCGCAAG - 3’ 5’ – CACCGGTTTCTGCTAATTGACG – 3’		
Virulence factor	*ebpC*	359	*E. faecalis* MMH 594	*Enterococcus* spp.		5’ – CGGCACAGCTCGTGATTAC – 3’ 5’ – CGCCACCACCATATTCGTAGC -3’		

Virulence factor	*agg*	775	*E. faecalis* MMH 594	*Enterococcus* spp.	Aggregation substance	5’ – CGGTACAGTTGGCAGTGTTTCG – 3’ 5’ – GGCTTGTGGGTCTTTGGCAGAG -3’		Lemsaddek *et al.* unpublished
Virulence factor	*gelE*	419	*E. faecalis* MMH 594	*Enterococcus* spp.	Gelatinase	5’ – ACCCCGTATCATTGGTTT -3’ 5’ – ACGCATTGCTTTTCCATC – 3’		Eaton and Gasson, 2001
Virulence factor	*cylA*	628	*E. faecalis* MMH 594	*Enterococcus* spp.	Cytolysina	5’ – CGGGGATTGATAGGCTTCDSMZ - 3’ 5’ – TAACCATCTGGAAAGTCAGCAG – 3		Carlos *et al.*, 2010
Virulence factor	*epa*	486	*E. faecalis* MMH 594	*Enterococcus* spp.	enterococcal polysaccharide antigen gene	5’ – ATTACTDSMZATCGCGCTGAAG – 3’ 5’ – GAGADSMZGATAGCCTGCTTTG – 3’		Lemsaddek *et al.* unpublished
Virulence factor	*efaAfs*	534	*E. faecalis* MMH 594	*Enterococcus* spp.	adhesin-like *E. faecalis* endocarditis antigen - $efaA_{fs}$	5’ – GGCTTCTGGTGCGACGATTG – 3’ 5´- AAGCATGCGGATCTTCTGTTTG – 3’		Carlos *et al.*, 2010
Virulence factor	*frsB*	327	*E. faecalis* MMH 594	*Enterococcus* spp.	Regulationof expression *gelE*	5’ – TTTACGGCCTGTCGCAGGTG - 3’ 5’ – CCTTGGATGACGAGACCGTAG – 3’		Lemsaddek *et al.* unpublished
Antibiotic susceptibility	*erm(B)*	639	*E. faecalis V583, E. faecalis AR01/DG*	*Enterococcus* spp.	Erythromycin resistance gene	5’ – GAAAAGGTACTCAACCAAATA – 3’ 5’ - AGTAACGGTACTTAAATTGTTTAC – 3’	94 ºC (5 min), 35 cycles of 94 ºC (30 sec), 57 ºC (30 sec), 72 ºC (30 sec) 72 ºC (5 min) 4 ºC (infinite)	Macovei and Zurek 2006
Antibiotic susceptibility	*tet(M)*	155	*E. faecalis AR01/DG*	*Enterococcus* spp.	Tetracyclin resistance gene	5’ – ACAGAAAGCTTATTATATAAC – 3’ 5’ – TGGCGTGTCTATGATGTTCAC – 3’		Aminov *et al.*, 2001

Antibiotic susceptibility	*vanA*	931	*E. faecalis AR01/DG*	*Enterococcus* spp.	Vancomycin resistance gene	5’ - TTGGGGGTTGCTCAGAGGAG – 3’ 5’ - CTTCGT TCAGTACAATGCGG – 3’		Yean *et al.*, 2007
Antibiotic susceptibility	*vanB*	536	*E. faecalis V583*	*Enterococcus* spp.		5’ - AAGCTATGCAAGAAGCCA TG – 3’ 5´- CCGACA ATCAAATCDSMZTC – 3’		Elsayed *et al.*, 2001

Appendix B

Class		Antibiotic	Symbol	Disc concentration	CLSI Breackpoints (mm)		
					S	I	R
Aminoglycosides		Gentamicin	CN	10 µg	≥ 15	13-14	≤ 12
		Gentamicin	CN	120 µg	≥ 10	7-9	6
		Streptomycin	S	10 µg	≥ 15	12-14	≤ 11
β - Lactams	Penicillin	Ampicillin	AMP	10 µg	≥ 17	n.a	≤ 16
		Amoxycillin/ Clavulanate	AMC	30 µg	≥ 18	14-17	≤ 13
		Penicillin G	P	10 units	≥ 15	n.a	≤ 14
	Cephalosporin	Cefalexin	CL	30 µg	≥ 18	15-17	≤ 14
		Cephotaxim	CTX	30 µg	≥ 23	15-22	≤ 14
Phenicols		Chloramphenicol	C	30 µg	≥ 18	13-17	≤ 12
Glycopeptides		Vancomycin	VAN	30 µg	≥ 17	15-16	≤ 14
Macrolides		Erythromycin	E	15 µg	≥ 23	14-22	≤ 13
Quinolones		Nalidixic Acid	NA	30 µg	≥ 19	14-18	≤ 13
Sulphonamides/ Pyrimidines		Sulphamethoxazole/ Trimethropim	STX	25 µg	≥ 19	16-15	≤ 15
Tetracyclines		Tetracycline	TET	30 µg	≥ 19	15-18	≤ 14